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(57) Abstract			
<p>The present invention relates to cleaning compositions comprising protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions correspond to naturally-occurring subtilisin from <u>Bacillus amyloliquefaciens</u> or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as <u>Bacillus licheniformis</u> subtilisin).</p>			

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PROTEASE-CONTAINING CLEANING COMPOSITIONS

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This application is a continuation-in-part application of U.S. Application
15 Serial No. 08/136,797, filed October 14, 1993 and U.S. Application Serial No.
08/237,938, filed May 2, 1994, both incorporated herein by reference in their
entirety.

TECHNICAL FIELD

The present invention relates to a variety of cleaning compositions
20 comprising novel protease enzymes which are carbonyl hydrolase variants.

BACKGROUND

Enzymes make up the largest class of naturally occurring proteins.
Each class of enzyme generally catalyzes (accelerates a reaction without being
consumed) a different kind of chemical reaction. One class of enzymes, known
25 as proteases, are known for their ability to hydrolyze (break down a compound
into two or more simpler compounds with the uptake of the H and OH parts of a
water molecule on either side of the chemical bond cleaved) other proteins.
This ability to hydrolyze proteins has been taken advantage of by incorporating
naturally occurring and protein engineered proteases as an additive to laundry
30 detergent preparations. Many stains on clothes are proteinaceous and wide-
specificity proteases can substantially improve removal of such stains.

Unfortunately, the efficacy level of these proteins in their natural,
bacterial environment, frequently does not translate into the relatively unnatural
wash environment. Specifically, protease characteristics such as thermal
35 stability, pH stability, oxidative stability and substrate specificity are not
necessarily optimized for utilization outside the natural environment of the
enzyme.

The amino acid sequence of the protease enzyme determines the characteristics of the protease. A change of the amino acid sequence of the protease may alter the properties of the enzyme to varying degrees, or may even inactivate the enzyme, depending upon the location, nature and/or

5 magnitude of the change in the amino acid sequence. Several approaches have been taken to alter the amino acid sequence of proteases in an attempt to improve their properties, with the goal of increasing the efficacy of the protease for cleaning uses such as in the wash environment. These approaches include altering the amino acid sequence to enhance thermal stability and to improve
10 oxidation stability under quite diverse conditions.

Despite the variety of approaches described in the art, there is a continuing need for new effective variants of proteases useful for cleaning a variety of surfaces. It is therefore an object of the present invention to provide cleaning compositions containing protease enzymes which are carbonyl
15 hydrolase variants having improved proteolytic activity, substrate specificity, stability and/or enhanced performance characteristics. These and other objects will become readily apparent from the detailed description which follows.

SUMMARY OF THE INVENTION

20 The present invention relates to cleaning compositions comprising:
(a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions corresponds to naturally-occurring subtilisin from
25 Bacillus amyloliquefaciens or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as Bacillus lentus subtilisin); and
30 (b) one or more cleaning composition materials compatable with the protease enzyme.

The present invention also relates to methods for cleaning items in need of cleaning by contacting said item with a protease enzyme which is a carbonyl hydrolase variant as described herein. The invention therefore encompasses a method for cleaning fabrics comprising contacting, preferably with agitation,

said fabrics with an aqueous liquor containing said protease enzyme. The method can be carried out at temperatures below about 60°C but, of course, is quite effective at laundry temperatures up to the boil. The present invention also relates to a method for cleaning dishes by contacting a dish in need of 5 cleaning with a protease enzyme as described herein. The present invention methods also include methods for personal cleansing, said methods comprising contacting the part of the human or lower animal body in need of cleaning with a protease enzyme as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No.6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN') and *Bacillus lentinus* (wild-type).

15 Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No.7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid 20 sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentinus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

25 Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

30 Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentinus* (Seq. ID No.11). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

35 Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103A/V104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

Fig. 10 shows the stability of a preferred mutant enzyme compared to wild-type, in a liquid detergent formulation.

DETAILED DESCRIPTION OF THE INVENTION

5 1. Protease Enzymes:

The invention includes protease enzymes which are non-naturally-occurring carbonyl hydrolase variants having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. The precursor carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. The plurality of amino acid residues of the precursor enzyme correspond to position +76 in combination with one or more of the following residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins, such as *Bacillus lenthus* subtilisin.

The carbonyl hydrolase variants which are protease enzyme useful in the present invention compositions comprise replacement of amino acid residue +76 in combination with one or more additional modifications.

25 Preferably the variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 30 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably 35 the variant enzymes useful for the present invention comprise the substitution, deletion or insertion of an amino acid residue in the following combination of

residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104 of *B. amyloliquefaciens* subtilisin.

Variant DNA sequences encoding such carbonyl hydrolase or subtilisin variants are derived from a precursor DNA sequence which encodes a naturally-occurring or recombinant precursor enzyme. The variant DNA sequences are derived by modifying the precursor DNA sequence to encode the substitution of one or more specific amino acid residues encoded by the precursor DNA sequence corresponding to positions 76, 99, 101, 103, 104, 107, 123, 27, 105, 109, 126, 128, 135, 156, 166, 195, 197, 204, 206, 210, 216, 217, 218, 222, 260, 265 and/or 274, in *Bacillus amyloliquefaciens* or any combination thereof. Although the amino acid residues identified for modification herein are identified according to the numbering applicable to *B. amyloliquefaciens* (which has become the conventional method for identifying residue positions in all subtilisins), the preferred precursor DNA sequence useful for the present invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 6 (Seq. ID No. 11).

These variant DNA sequences encode the insertion or substitution of the amino acid residue 76 in combination with one or more additional modification. Preferably the variant DNA sequences encode the substitution or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably the variant DNA sequences encode for the modification of the following combinations of residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These recombinant DNA sequences encode carbonyl hydrolase variants having a novel amino acid sequence and, in general, at least one property which is substantially different from the same property of the enzyme encoded by the precursor carbonyl hydrolase DNA sequence. Such properties include proteolytic activity, substrate specificity, stability, altered pH profile and/or enhanced performance characteristics.

The protease enzymes useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application 5 reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, J.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

Preferably, the substitution to be made at each of the identified amino acid residue positions include but are not limited to: substitutions at position 10 76 including D, H, E, G, F, K, P and N; substitutions at position 99 including D, T, N, Q, G and S; substitutions at position 101 including G, D, K, L, A, E, S and R; substitutions at position 103 including Q, T, D, E, Y, K, G, R, S, and A; substitutions at position 104 including all nineteen naturally-occurring amino acids; substitutions at position 107 including V, L, M, Y, G, E, F, T, S, A, N and I; substitutions at position 123 including N, T, I, G, A, C, and S; substitutions at position 27 including K, N, C, V and T; substitutions at position 105 including A, D, G, R and N; substitutions at position 107 including A, L, V, Y, G, F, T, S and A; substitutions at position 109 including S, K, R, A, N and D; substitutions at position 126 including A, F, I, V and G; substitutions at position 128 including 15 G, L and A; substitutions at position 135 including A, F, I, S and V; substitutions at position 156 including D, E, A, G, Q and K; substitutions at position 166 including all nineteen naturally-occurring amino acids; substitutions at position 195 including E; substitutions at position 197 including E; substitutions at position 204 including A, G, C, S and D; substitutions at position 206 including 20 L, Y, N, D and E; substitutions at position 210 including L, I, S, C and F; substitutions at position 216 including V, E, T and K; substitutions at position 217 including all nineteen naturally-occurring amino acids; substitutions at position 218 including S, A, G, T and V; substitutions at position 222 including all nineteen naturally-occurring amino acids; substitutions at position 260 including P, N, G, A, S, C, K and D; substitutions at position 265 including N, G, A, S, C, K, Y and H; and substitutions at position 274 including A and S. The specifically preferred amino acid(s) to be substituted at each such position are designated below in Table I. Although specific amino acids are shown in 25 Table I, it should be understood that any amino acid may be substituted at the identified residues.

Table I

Amino Acid

Preferred Amino Acid to

	<u>Residue</u>	<u>be Substituted/Inserted</u>
	+76	D,H
	+99	D,T,N,G
	+101	R,G,D,K,L,A,E
5	+103	A,Q,T,D,E,Y,K,G,R
	+104	I,Y,S,L,A,T,G,F,M,W,D,V,N
	+107	V,L,Y,G,F,T,S,A,N
	+123	S,T,I
	+27	K
10	+105	A,D
	+109	S,K,R
	+126	A,I,V,F
	+128	G,L
	+135	I,A,S
15	+156	E,D,Q
	+166	D,G,E,K,N,A,F,I,V,L
	+195	E
	+197	E
	+204	A,G,C
20	+206	L
	+210	I,S,C
	+216	V
	+217	H,I,Y,C,A,G,F,S,N,E,K
	+218	S
25	+222	A,Q,S,C,I,K
	+260	P,A,S,N,G
	+265	N,A,G,S
	+274	A,S

These protease enzymes containing *in vitro* mutations in *B. lento*s 30 subtilisin at an amino acid residue equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin produces subtilisin variants exhibiting altered stability (e.g., modified autoproteolytic stability) over precursor subtilisins. (See Tables IV and VI.)

Also, *in vitro* mutation at residues equivalent to +99, +101, +103, +104, 35 +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin, alone or in combination with each other

and in any combination with +76 mutations, produce subtilisin variants exhibiting altered proteolytic activity, altered thermal stability, alt red pH profile, alt red substrate specificity and/or altered performance characteristics.

Carbonyl hydrolases are protease enzymes which hydrolyze compounds 5 containing



bonds in which X is oxygen or nitrogen. They include naturally-occurring 10 carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, 15 carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, 20 insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258, the disclosure of which are incorporated herein by reference.

Subtilisins are bacterial or fungal carbonyl hydrolases which generally 25 act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the 30 same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related 35 proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however, is histidine-aspartate-serine. Thus,

subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many prokaryotic and eucaryotic organisms. Suitable examples of prokaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, bovine sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with prokaryotic and eucaryotic sources.

A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases (such as a subtilisin) include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases (subtilisin). The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as

methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific residues corresponding to position +76 in combination with one or more of the following positions +99, +101, +103, +104, +107, +123, +27, 5 +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 of *Bacillus amyloliquefaciens* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 10 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 15 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222; and most preferably are 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The protease 20 enzymes useful in the present invention, however, are not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. Preferably, the precursor subtilisin is *Bacillus lenthus* subtilisin and the 25 substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lenthus* corresponding to those listed above.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to 30 a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of 35 residues known to be invariant in subtilisins for which sequence is known. Fig. 2 herein shows the conserved residues as between *amyloliquefaciens* subtilisin

and *B. lenth* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of 5 *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

10 For example, in Fig. 3 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lenth* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in 15 each sequence. These conserved residues (as between BPN' and *B. lenth*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lenth* 20 (PCT Publication No. W089/06279 published July 13, 1989), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP O 328 299), which is highly homologous to the preferred *Bacillus lenth* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce 25 the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lenth* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lenth* and *B. licheniformis*.

30 Thus, for example, the amino acid at position +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lenth* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of all the amino acid residues identified herein for substitution 35 versus the preferred substitution for each such position is provided in Table II for illustrative purposes.

Table II

	+76	+99	+101	+103	+104	+107	+123
<i>B. amyloliquefaciens</i> (wild-type)	N	D	S	Q	Y	I	N
5 <i>B. lenthus</i> (wild-type)	N	S	S	S	V	I	N

Most Preferred Substitution D D R A I/Y V S

Equivalent residues may also be defined by determining homology at the 10 level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. 15 Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R 20 factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_b |F_o(b)| - |F_c(b)|}{\sum_b |F_o(b)|}$$

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate 25 binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy 30 the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO 35 Publication No. 0 251 446 (equivalent to U.S. Patent Application SN

08/212,291, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are 5 conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved 10 residues, such replacements should not result in a naturally-occurring sequence. The carbonyl hydrolase variants useful in the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

15 "Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are 20 expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In the Examples, the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) is used.

25 A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by 30 the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The protease enzymes useful for the present invention utilize such sequences to effect the secretion of the carbonyl hydrolase variants as described herein. A preferred signal 35 sequence used in the Examples comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

5 "Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA

10 ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification,

15 "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, included herein are such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are prokaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in U.S. Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in U.S. Patent 5,264,366. Other host cells for expressing subtilisin include *Bacillus subtilis* I168 (also described in U.S. Patent 4,760,025 (RE 34,606) and U.S. Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

30 Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the carbonyl hydrolase variants or expressing the desired carbonyl hydrolase variant. In the case of vectors which encode the pre- or prepro-form of the carbonyl hydrolase variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked," when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein

5 most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes.

10 Positively hybridizing clones are then mapped and sequenced. The *B. latus* gene used in the Examples is cloned as described in Example 1 of U.S. Patent 5,185,258, the disclosure of which is incorporated herein. The BPN' gene used in the Examples is cloned as described in Example 1 in RE 34,606, the disclosure of which is incorporated herein.

15 The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the

20 gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the hydrolase gene and, desirably, a selection gene such

25 as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate

30 multiple copies of the hydrolase gene into host genome. This is facilitated by prokaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

35

The genes used in the present examples are a natural *B. lenthus* gene and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of U.S. Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in U.S. Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants useful in the present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in

accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different 5 restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the 10 restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

15 As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant 20 enzymes of the present invention may have other modified properties such as K_m , K_u , K_u/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, for hydrolytic processes such as laundry uses.

25 One objective can be to secure a variant carbonyl hydrolase having altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are variant 30 enzymes having altered thermal stability and/or altered substrate specificity as compared to the precursor. Preferably the carbonyl hydrolase to be mutated is a subtilisin. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 or any 35 combination thereof in *Bacillus amyloliquefaciens* subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. Conversely, in some instances it may be desirable to increase the proteolytic

activity of the variant enzyme versus its precursor. Additionally, increases or decreases (alteration) of the stability of the variant, whether alkaline or thermal stability, may be desirable. Increases or decreases in K_m , K_m or K_m/K_m are specific to the substrate used to determine these kinetic parameters.

5 Also, it has been determined that residues equivalent to +76 in combination with a number of other modifications in subtilisin are important in modulating overall stability and/or proteolytic activity of the enzyme. Thus, as set forth in the Examples, the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 can be substituted with Aspartate (D) in the preferred 10 protease enzymes in combination with modification of one or more of the following amino acid residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

15 The most preferred protease enzymes useful in this invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/S99D; N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V and N76D/S101R/S103A/V104I. These substitutions are preferably made in 20 *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* subtilisin.

Based on the results obtained with this and other variant subtilisins, it is apparent that residues in carbonyl hydrolases (preferably subtilisin) equivalent to positions +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, 25 +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in *Bacillus amyloliquefaciens* are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

30 The following is presented by way of example for manufacturing protease enzymes useful in the present invention compositions.

Protease Manufacture Example

Construction for the Expression of GG36 Gene in *B. subtilis*

The cloning and the construction for expression of the subtilisin gene from *B. lentus* is performed essentially the same as that described in U.S. Patent 35 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) is further modified in the following manner, as shown in Fig. 5. The PstI site that is introduced during the construction of the GGA274 plasmid is removed by the

oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5' GAAGCTGCAACTCGTTAAA 3' (Seq. ID No.1). The underlined "A" residue eliminates the recognition sequence of restriction enzyme PstI and changes the corresponding amino acid residue 5 from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lenthus* subtilisin gene sequences. The DNA segment encoding subtilisin is excised from the plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment is subcloned back into Bacteriophage M13-based vectors, such 10 as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, are performed to move the mutated subtilisin gene back 15 into an expression plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

Oligonucleotide-Directed Mutagenesis

15 Oligonucleotide-directed mutagenesis is performed as described in Zolier, M. et al. (1983), *Methods Enzymol.*, 100:468-500. As an example, a synthetic oligonucleotide of the sequence 5' GCTGCTCTAGACAATTG 3' (Seq. ID No.2) is used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" 20 residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC changes asparagine at +76 to aspartate.

25 For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the combination of mutations desired. For example, an oligonucleotide of the sequence 5' GTATTAGGGCGGACGGTCGAGGCGCCATCAGCTCGATT 3' (Seq. ID No.3) is used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the 30 sequence 5' TCAGGTTCGGTTCGAGGCGTTGCCAAGGATTG 3' (Seq. ID No.4) and 5' CACGTTGCTAGCTGAGTTAG 3' (Seq. ID No.5) are utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produce desired changes either in amino acid sequences or restriction enzyme recognition sequences.

35 Proteolytic Activity of Subtilisin Variants

Following the methods of Oligonucleotide-Directed Mutagenesis hereinbefore, the variants listed in Table III are made. Proteolytic activity of

each of these subtilisin variants is shown in Table III. The kinetic parameters k_m , K_m , and k_m/K_m are measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p.

- 5 1030. Briefly, a small aliquot of subtilisin variant stock solution is added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at 25°C. The reaction progress is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm. Kinetic parameters are obtained by using a non-linear
- 10 10 regression algorithm to fit the reaction velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table III

Kinetic Parameters k_{cat} , K_M and k_{cat}/K_M
Measured for Bacillus lenthus Subtilisin and Variants

<u>Protease #</u>	<u>Enzyme</u> <u>Variants</u>	<u>k_{cat} (s⁻¹)</u>	<u>K_M (M)</u>	<u>k_{cat}/K_M</u> (s ⁻¹ M ⁻¹)
-	<u>B. lenthus</u> Subtilisin	170	0.00078	2.18×10^5
-	N76D	219	0.0008	2.74×10^5
1	N76D/S99D	88	0.00061	1.44×10^5
2	N76D/S101R	371	0.0013	2.85×10^5
3	N76D/S103A	400	0.0014	2.86×10^5
4	N76D/V104I	459	0.0011	4.17×10^5
5	N76D/I107V	219	0.0011	1.99×10^5
6	N76D/N123S	115	0.0018	6.40×10^4
7	N76D/S99D/S101R	146	0.00038	3.84×10^5
8	N76D/S99D/S103A	157	0.0012	1.31×10^5
9	N76D/S99D/V104I	247	0.00097	2.55×10^5
10	N76D/S101R/S103A	405	0.00069	5.90×10^5
11	N76D/S101R/V104I	540	0.00049	1.10×10^6
12	N76D/S103A/V104I	832	0.0016	5.20×10^5
13	N76D/V104I/I107V	497	0.00045	1.10×10^6
14	N76D/V104Y/I107V	330	0.00017	1.90×10^6
15	N76D/V104I/N123S	251	0.0026	9.65×10^4
16	N76D/I107V/N123S	147	0.0035	4.20×10^4
17	N76D/S99D/S101R/S103A	242	0.00074	3.27×10^5
18	N76D/S99D/S101R/V104I	403	0.00072	5.60×10^5
19	N76D/S99D/S103A/V104I	420	0.0016	2.62×10^5
20	N76D/S101R/S103A/V104I	731	0.00065	1.12×10^6
21	N76D/S103A/V104I/N123S	321	0.0026	1.23×10^5
22	N76D/V104I/I107V/N123S	231	0.003	7.70×10^4
23	N76D/S99D/S101R/S103A/V104I	624	0.00098	6.37×10^5
24	N76D/S99D/S103A/V104I/N123S	194	0.0043	4.51×10^4
25	N76D/S99D/S101R/S103A/V104I/N123S	311	0.0023	1.35×10^5

The results listed in Table III indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal 5 that proteolytic activity is significantly altered for *Bacillus lenthus* subtilisin by the various combinations of substitutions at amino acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and 123 in *Bacillus amyloliquefaciens*.

Thermal Stability of Subtilisin Variants

A comparison of thermal stability observed for *Bacillus lenthus* subtilisin and the variants of the present invention made by the process of Oligonucleotide-Directed Mutagenesis hereinbefore is shown in Table IV.

Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or

5 without 50 mM CaCl₂, is aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes are then placed on ice for 10 minutes. Aliquots from the tubes are assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M

10 tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table IV indicate that a vast majority of variants exhibit thermal stability comparable to

15 *Bacillus lenthus* subtilisin (24 out of 26) in the test condition with 50mM CaCl₂ added. In the test condition without 50mM CaCl₂ added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lenthus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and

20 N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM CaCl₂ added.

Table IVThermal Stability Measured for *Bacillus lenthus* Subtilisin and VariantsAt pH 10, 60°C, +/- 50mM CaCl₂ Added

Enzyme	% Initial Activity Remaining	
	-CaCl ₂	+CaCl ₂
<i>B. lenthus</i> Subtilisin	2	96
N76D	34	97
N76D/S99D	49	98
N76D/S101R	0	82
N76D/S103A	26	92
N76D/V104I	58	98
N76D/I107V	32	96
N76D/N123S	0	97
N76D/S99D/S101R	30	100
N76D/S99D/S103A	36	100
N76D/S99D/V104I	48	97

N76D/S101R/S103A	26	100
N76D/S101R/V104I	38	100
N76D/S103A/V104I	58	100
N76D/V104I/I107V	60	97
N76D/V104Y/I107V	48	74
N76D/V104I/N123S	0	98
N76D/I107V/N123S	16	100
N76D/S99D/S101R/S103A	38	100
N76D/S99D/S101R/V104I	33	100
N76D/S99D/S103A/V104I	38	98
N76D/S101R/S103A/V104I	40	99
N76D/S103A/V104I/N123S	1	98
N76D/V104I/I107V/N123S	3	99
N76D/S99D/S101R/S103A/V104I	36	99
N76D/S99D/S103A/V104I/N123S	2	95
N76D/S99D/S101R/S103A/V104I/N123S	0	100

Oligonucleotide-Directed Mutagenesis with
Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lenthus* Variants

The mutagenesis protocol is essentially the same as described above in

5 Oligonucleotide-Directed Mutagenesis. The single-stranded DNA template is generated by phagemid method. To construct the phagemid vector for generating the single-stranded DNA template we first construct the vector pBCDAICAT. The flow chart of vector construction is outlined in Figure 8. First, the *C1a1* to *C1a1* fragment encoding the CAT gene from pC194 plasmid

10 (Horinouchi, S. and Weisblum, B., J. Bacteriol., 150:8-15, 1982) is cloned into the *Acc1* site of polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make plasmid pUCCHL and the *EcoRI-Dra1* 0.6 KB fragment from the 5' end of the GG36DAI encoding DNA is cloned into the *EcoRI* and *EcoRV* sites of pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The

15 single *EcoRI* site of the plasmid pBC2SK5 is eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI-Dra1* fragment which is cloned into pBCSK-5R is isolated as a *PstI-HindIII* fragment and cloned into the *PstI-HindIII* site of the pUCCHL (part of the

20 polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene is excised as an *EcoRI-BamHI* fragment and

cloned into the *EcoRI-BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI-HindIII* fragment of pUCCAT is then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

To generate single-stranded DNA, *E. coli*-containing pBCDAICAT are 5 infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE 45:333-338, 1986. Once the single-stranded DNA template is available, standard mutagenesis methods as described above in Oligonucleotide-Directed Mutagenesis are carried out. The construction of certain mutants is detailed 10 below for illustrative purposes.

For the construction of *B. latus* (GG36) N76D/S103A/Y104I/L217H, an *EcoRI-BamHI* DNA fragment encoding GG36 N76D/S103A/Y104I is used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT.

After the single-stranded DNA template is made following the protocol 15 described above, a mutagenesis primer with the following sequence

* * * * x C1a/

5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)

is used to make the L217H. As before, the underlined residues denote the 20 nucleotide changes that are made and the x C1a/ indicates that the existing C1a/ site is eliminated after the mutagenesis. The mutagenesis protocol is as described in Oligonucleotide-Directed Mutagenesis hereinbefore. After the mutagenesis, plasmid DNA is first screened for the elimination of the C1a/ site and those clones missing the C1a/ site are subjected to DNA sequence 25 analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

B. Construction of BPN' Variants and their Expression in *B. subtilis*

The construction of *B. amyloliquefaciens* (BPN')

N76D/Q103A/Y104I/Y217L is done in a similar fashion except in two 30 consecutive steps. N76D is first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis is done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI-BamHI* fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., 35 PNAS, 84, 5167, 1087) is used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L is used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA is generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

* * * * * *Xba*I

5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3' (Seq. ID
No.14)

5 is used to generate the change N76D. Single-stranded DNA is then prepared
from the pBCFNACAT plasmid containing the BPN' N76D/Y217L (the
pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another
oligonucleotide with the sequence

* * * * * *Pvu*II

5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3' (Seq. ID
10 No.15)

10 to obtain BPN' N76D/Q103A/Y104I/Y217L. All steps involved in the cloning,
the single-stranded DNA preparation, the mutagenesis, and the screening for
mutants are carried out as described above. Expression of the BPN' gene and
its variants are achieved by integrating plasmid DNA (pBCFNACAT containing
15 the different variants' BPN' gene) directly into a protease-deficient strain of
Bacillus subtilis as described in RE 34,606.

15 Numerous variants are made as per the teachings of these Protease
Manufacture Examples. Kinetics data and stability data are generated for such
variants. The kinetics data are generated using the methods described
20 hereinbefore and are provided in Table V. The stability data are generated as
detailed herein. Results are shown in Table VI.

Thermal Stability Assay Procedure

25 Purified enzyme is buffer-exchanged into 0.1 M glycine pH 10.0, 0.01%
Tween-80 by applying the enzyme to a column consisting of Sephadex G-25
equilibrated with this buffer and eluting the enzyme from the column using the
same buffer.

30 To a tube containing 0.1 M glycine, 0.01% Tween-80 pH 10.0
thermostatted at 60°C, the buffer-exchanged enzyme is added to give a final
enzyme concentration of 15 ug/ml.

35 Aliquots are removed from the 60°C incubation at various times and
immediately assayed for enzyme activity by addition to a 1 cm cuvette
containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-
Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6,
thermostatted at 25°C. The initial linear reaction velocity is followed
spectrophotometrically by monitoring the absorbance of the reaction product p-
nitroaniline at 410 nm as a function of time.

Half-life, which is the length of time required for 50% enzyme
inactivation, is determined from the first-order plot of reaction velocity as a
function of the time of incubation at 60°C.

The data are presented in Table VI as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table V

Enzyme	kcat (s ⁻¹)	KM (mM)	kcat/KM (s ⁻¹ M ⁻¹)
B. lentus subtilisin	170	0.78	2.20E+05
N76D/S103G/V104I*	380	1.4	2.70E+05
N76D/S103A/V104F	730	0.33	2.20E+06
N76D/S103A/V104N	790	2.8	2.80E+05
N76D/S103A/V104S	170	0.83	2.00E+05
N76D/S103A/V104T	370	1.9	2.00E+05
N76D/S103A/V104W	880	0.31	2.80E+06
N76D/S103A/V104Y	690	0.5	1.40E+06
K27R/N76D/V104Y/N123S	500	1.2	4.20E+05
N76D/S101G/S103A/V104I*	620	1.3	4.80E+05
N76D/S103A/V104I/S105A*	550	1.3	4.20E+05
N76D/S103A/V104I/S105D*	440	1.7	2.60E+05
N76D/S103A/V104T/I107A*	120	5.7	2.10E+04
N76D/S103A/V104T/I107L*	310	3.2	9.70E+04
N76D/S103A/V104I/L126A	90	2.2	4.10E+04
N76D/S103A/V104I/L126F	180	1.9	9.50E+04
N76D/S103A/V104I/L126I	100	2.4	4.20E+04
N76D/S103A/V104I/L126V	64	3.2	2.00E+04
N76D/S103A/V104I/S128G*	560	1.7	3.30E+05
N76D/S103A/V104I/S128L*	430	3.8	1.10E+05
N76D/S103A/V104I/L135A	140	0.76	1.80E+05
N76D/S103A/V104I/L135F	390	0.69	5.70E+05
N76D/S103A/V104I/L135I	110	0.73	1.50E+05
N76D/S103A/V104I/L135V	140	0.86	1.60E+05
N76D/S103A/V104I/S156E*	170	2.6	6.50E+04
N76D/S103A/V104I/S166D*	160	3.5	4.60E+04
N76D/S103A/V104I/D197E	510	1.4	3.60E+05
N76D/S103A/V104I/N204A*	530	1.1	4.80E+05
N76D/S103A/V104I/N204G*	580	1.4	4.10E+05
N76D/S103A/V104I/N204C*	370	1.3	2.90E+05
N76D/S103A/V104I/P210I*	500	1.2	4.20E+05
N76D/S103A/V104I/L217H*	80	0.63	1.30E+05
N76D/S103A/V104I/M222A	70	3.1	2.30E+04
N76D/S103A/V104I/M222S	80	3.1	2.60E+04
N76D/S103A/V104I/T260P	660	1.5	4.40E+05
N76D/S103A/V104I/S265N	590	1.3	4.50E+05
K27R/N76D/V104Y/I107V/N123S	220	1.4	1.60E+05
K27R/N76D/V104Y/N123S/D197E	430	1.1	3.90E+05
K27R/N76D/V104Y/N123S/N204C	400	1.1	3.60E+05
K27R/N76D/V104Y/N123S/Q206L	440	1.2	3.70E+05

K27R/N76D/V104Y/N123S/S216V	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/N218S	760	0.98	7.80E+05
K27R/N76D/V104Y/N123S/T260P	410	1.2	3.40E+05
K27R/N76D/V104Y/N123S/T274A	390	1	3.90E+05
N76D/S103A/V104I/L126F/S265N	170	2.1	8.10E+04
N76D/S103A/V104I/S156E/S166D*	40	6.3	6.40E+03
K27R/N76D/V104Y/N123S/G195E/G197E	410	0.98	4.20E+05
K27R/N76D/V104Y/N123S/G195E/N218S	540	0.66	8.20E+05
K27R/N76D/V104Y/N123S/D197E/N218S	770	0.79	9.80E+05
K27R/N76D/V104Y/N123S/N204C/N218S	610	0.99	6.20E+05
K27R/N76D/V104Y/N123S/Q206L/N218S	580	0.78	7.40E+05
K27R/N76D/V104Y/N123S/N218S/T260P	660	1	6.60E+05
K27R/N76D/V104Y/N123S/N218S/T274A	590	0.89	6.60E+05
K27R/N76D/V104Y/Q109S/N123S/N218S/T274A	520	1	5.20E+05
K27R/N76D/V104Y/N123S/G195E/D197E/N218S	460	0.65	7.10E+05
B. amyloliquefaciens subtilisin (BPN')	50	0.14	3.60E+05
BPN'-N76D/Y217L*	380	0.46	8.30E+05

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

5

Table VI
Enzyme **Thermal Stability**
 (% half-life of native enzyme)

B. lenthus subtilisin	100
N76D	590
N76D/S99D	840
N76D/S103A	390
N76D/V104I	660
N76D/I107V	710
N76D/N123S	70
N76D/S99D/S101R	610
N76D/S99D/S103A	590
N76D/S99D/V104I	910
N76D/S101R/S103A	930
N76D/S101R/V104I	500
N76D/S103A/V104I	460
N76D/S103G/V104I*	370
N76D/S103A/V104F	480
N76D/S103A/V104N	230
N76D/S103A/V104S	230
N76D/S103A/V104T	370

N76D/S103A/V104W	280
N76D/S103A/V104Y	400
N76D/V104I/I107V	940
N76D/V104Y/I107V	820
N76D/V104I/N123S	80
N76D/I107V/N123S	150
K27R/N76D/V104Y/N123S	100
N76D/S99D/S101R/S103A	570
N76D/S99D/S101R/V104I	1000
N76D/S99D/S103A/V104I	680
N76D/S101G/S103A/V104I*	390
N76D/S101R/S103A/V104I	470
N76D/S103A/V104I/S105A*	360
N76D/S103A/V104I/S105D*	370
N76D/S103A/V104T/I107A*	270
N76D/S103A/V104T/I107L*	230
N76D/S103A/V104I/N123S	110
N76D/V104I/I107V/N123S	220
N76D/S103A/V104I/L126A	270
N76D/S103A/V104I/L126F	950
N76D/S103A/V104I/L126I	410
N76D/S103A/V104I/L126V	320
N76D/S103A/V104I/S128G*	640
N76D/S103A/V104I/S128L*	760
N76D/S103A/V104I/L135A	230
N76D/S103A/V104I/L135F	200
N76D/S103A/V104I/L135I	510
N76D/S103A/V104I/L135V	500
N76D/S103A/V104I/S156E*	120
N76D/S103A/V104I/S166D*	590
N76D/S103A/V104I/D197E	460
N76D/S103A/V104I/N204A*	230
N76D/S103A/V104I/N204G*	240
N76D/S103A/V104I/N204C*	500
N76D/S103A/V104I/P210I*	1370
N76D/S103A/V104I/L217H*	60
N76D/S103A/V104I/M222A	520
N76D/S103A/V104I/M222S	490
N76D/S103A/V104I/T260P	490
N76D/S103A/V104I/S265N	360
K27R/N76D/V104Y/I107V/N123S	210
K27R/N76D/V104Y/N123S/D197E	120
K27R/N76D/V104Y/N123S/N204C	110
K27R/N76D/V104Y/N123S/Q206L	380
K27R/N76D/V104Y/N123S/S216V	140
K27R/N76D/V104Y/N123S/N218S	270
K27R/N76D/V104Y/N123S/T260P	40
K27R/N76D/V104Y/N123S/T274A	60
N76D/S99D/S101R/S103A/V104I	590
N76D/S99D/S103A/V104I/N123S	110
N76D/S103A/V104I/L126F/S265N	810

N76D/S103A/V104I/S156E/S166D*	220
K27R/N76D/V104Y/N123S/G195E/G197E	90
K27R/N76D/V104Y/N123S/G195E/N218S	250
K27R/N76D/V104Y/N123S/D197E/N218S	270
K27R/N76D/V104Y/N123S/N204C/N218S	460
K27R/N76D/V104Y/N123S/Q206L/N218S	1400
K27R/N76D/V104Y/N123S/N218S/T260P	310
K27R/N76D/V104Y/N123S/N218S/T274A	180
N76D/S99D/S101R/S103A/V104I/N123S	90
K27R/N76D/V104Y/Q109S/N123S/N218S/T274	230
K27R/N76D/V104Y/N123S/G195E/D197E/N21	240
 B. amyloliquefaciens subtilisin (BPN')	100
BPN'-N76D/Y217L*	420

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

2. Cleaning Composition Materials:

5 The cleaning compositions of the present invention also comprise, in addition to the protease enzyme described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. The term "cleaning composition materials", as used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid; granule; spray composition), which materials are also compatible with the protease enzyme used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g., 10 through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the protease enzyme to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

15 An effective amount of one or more protease enzymes described above are included in compositions useful for cleaning a variety of surfaces in need of proteinaceous stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for cleaning fabrics, unlimited in form (e.g., 20 granular, liquid and bar formulations); dishwashing compositions (unlimited in form); oral cleaning compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); and denture cleaning compositions, unlimited in 25

form (e.g., liquid, tablet). As used herein, "effective amount of protease enzyme" refers to the quantity of protease enzyme described hereinbefore necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like.

Preferably the cleaning compositions of the present invention comprise from about 0.0001% to about 10% of one or more protease enzymes, more preferably from about 0.001% to about 1%, more preferably still from about 0.001% to about 0.1%. Several examples of various cleaning compositions wherein the protease enzymes may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless otherwise specified.

As used herein, "non-fabric cleaning compositions" include hard surface cleaning compositions, dishwashing compositions, oral cleaning compositions, denture cleaning compositions and personal cleansing compositions.

A. Cleaning Compositions for Hard Surfaces, Dishes and Fabrics

The protease enzymes can be used in any detergent composition where high sudsing and/or good insoluble substrate removal are desired. Thus the protease enzymes can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions and the like. Such compositions can be in the form of liquids, granules, bars and the like. Such compositions can be formulated as modern "concentrated" detergents which contain as much as 30%-60% by weight of surfactants.

The cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are typically present at levels of from about 0.1% to about 60%, preferably from about 1% to about 35%, of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C₁₁-C₁₈ alkyl benzene sulfonates and primary and random alkyl sulfates, the C₁₀-C₁₈ secondary (2,3) alkyl sulfates of the formulas CH₃(CH₂)_x(CHOSO₃⁻M⁺)CH₃ and CH₃(CH₂)_y(CHOSO₃⁻M⁺)CH₂CH₃ wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C₁₀-C₁₈ alkyl

alkoxy sulfates (especially EO 1-7 ethoxy sulfates), C₁₀-C₁₈ alkyl alkoxy carboxylates (especially the EO 1-7 ethoxycarboxylates), the C₁₀-C₁₈ alkyl polyglycosides, and their corresponding sulfated polyglycosides, C₁₂-C₁₈ alpha-sulfonated fatty acid esters, C₁₂-C₁₈ alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C₁₂-C₁₈ betaines and sulfobetaines ("sultaines"), C₁₀-C₁₈ amine oxides, C₈-C₂₄ sarcosinates (especially oleoyl sarcosinate), and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C₁₀-C₁₈ N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

Particularly useful is the class of nonionic surfactants which are condensates of ethylene oxide with a hydrophobic moiety to provide a surfactant having an average hydrophilic-lipophilic balance (HLB) in the range from 5 to 17, preferably from 6 to 14, more preferably from 7 to 12. The hydrophobic (lipophilic) moiety may be aliphatic or aromatic in nature and the length of the polyoxyethylene group which is condensed with any particular hydrophobic group can be readily adjusted to yield a water-soluble compound having the desired degree of balance between hydrophilic and hydrophobic elements. Especially preferred are the C₉-C₁₅ primary alcohol ethoxylates (or mixed ethoxy/propoxy) containing 3-8 moles of ethylene oxide per mole of alcohol, particularly the C₁₄-C₁₅ primary alcohols containing 6-8 moles of ethylene oxide per mole of alcohol, the C₁₂-C₁₅ primary alcohols containing 3-5 moles of ethylene oxide per mole of alcohol, and mixtures thereof.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C₁₀-C₁₆ alkylamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C₁₀-C₁₄ monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as MgCl₂, MgSO₄,

and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols

5 exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 10 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage 15 levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight.

20 Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases, peroxidases, and proteases in such 25 compositions, typically at levels of from about 0.001% to about 1% by weight. Various detergents and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 30 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Various soil release agents, especially of the anionic oligoester type, 35 various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated tetraethylene pentamine, various dispersing agents, especially

polyacrylates and polyasparatates, various brighteners, especially anionic brighteners, various dye transfer inhibiting agents, such as polyvinyl pyrrolidone, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays and clay flocculating polymers (e.g., poly(oxy ethylene)), and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions of the present invention. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

1. Hard surface cleaning compositions

As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 5%, more preferably still from about 0.001% to about 1% by weight of active protease enzyme of the composition. In addition to comprising one or more protease enzymes, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy/streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%. Preferably the pH should be in the range of about 8 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether,

diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are 5 typically present at levels of from about 0.5% to about 15%, preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as isopropanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" 10 application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

The hard surface cleaning composition embodiment of the present invention is illustrated by the following nonlimiting examples. (In the following examples, reference to "Protease #" in the examples is to the variant useful in 15 the present invention compositions having the given number in Table III hereinbefore.)

Examples 1-6

Liquid Hard Surface Cleaning Compositions

Example No.

20	Component	1	2	3	4	5	6
	Protease # 12	0.05	0.20	0.02	0.03	0.10	0.03
	Protease # 4	-	-	-	-	0.20	0.02
	EDTA**	-	-	2.90	2.90	-	-
	Na Citrate	-	-	-	-	2.90	2.90
25	NaC ₁₂ Alkyl-benzene sulfonate	1.95	-	1.95	-	1.95	-
	NaC ₁₂ Alkylsulfate	-	2.20	-	2.20	-	2.20
	NaC ₁₂ (ethoxy)*** sulfate	-	2.20	-	2.20	-	2.20
30	C ₁₂ Dimethylamine oxide	-	0.50	-	0.50	-	0.50
	Na Cumene sulfonate	1.30	-	1.30	-	1.30	-
	Hexyl Carbitol***	6.30	6.30	6.30	6.30	6.30	6.30
	Water****			balance to 100%			

35 **Na₄ ethylenediamine diacetic acid

***Diethyleneglycol monohexyl ether

****All formulas adjusted to pH 7

In Examples 1-4 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

5 In Examples 5 and 6, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 7-12

10 Spray Compositions for Cleaning Hard Surfaces
and Removing Household Mildew

	Example No.					
Component	7	8	9	10	11	12
Protease # 12	0.20	0.05	0.10	0.30	0.20	0.30
15 Protease # 4	-	-	-	-	0.30	0.10
Sodium octyl sulfate	2.00	2.00	2.00	2.00	2.00	2.00
Sodium dodecyl sulfate	4.00	4.00	4.00	4.00	4.00	4.00
Sodium hydroxide	0.80	0.80	0.80	0.80	0.80	0.80
Silicate (Na)	0.04	0.04	0.04	0.04	0.04	0.04
20 Perfume	0.35	0.35	0.35	0.35	0.35	0.35
Water	balance to 100%					

Product pH is about 7.

25 In Examples 7-10 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

30 In Examples 11 and 12, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

2. Dishwashing Compositions

35 In another embodiment of the present invention, dishwashing compositions comprise one or more protease enzymes. As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to, granular and liquid forms. The dishwashing composition embodiment of the present invention is illustrated by the following examples.

Examples 13-18
Dishwashing Composition

	<u>Component</u>	<u>Example No.</u>					
		13	14	15	16	17	18
5	Protease # 12	0.05	0.50	0.02	0.40	0.10	0.03
	Protease # 4	-	-	-	-	0.40	0.02
	C ₁₂ -C ₁₄ N-methyl-glucamide	0.90	0.90	0.90	0.90	0.90	0.90
	C ₁₂ ethoxy (1) sulfate	12.00	12.00	12.00	12.00	12.00	12.00
10	2-methyl undecanoic acid	4.50	4.50	-	4.50	4.50	-
	C ₁₂ ethoxy (2) carboxylate	4.50	4.50	4.50	4.50	4.50	4.50
	C ₁₂ alcohol ethoxylate (4)	3.00	3.00	3.00	3.00	3.00	3.00
	C ₁₂ amine oxide	3.00	3.00	3.00	3.00	3.00	3.00
	Sodium cumene sulfonate	2.00	2.00	2.00	2.00	2.00	2.00
15	Ethanol	4.00	4.00	4.00	4.00	4.00	4.00
	Mg ⁺⁺ (as MgCl ₂)	0.20	0.20	0.20	0.20	0.20	0.20
	Ca ⁺⁺ (as CaCl ₂)	0.40	0.40	0.40	0.40	0.40	0.40
	Water	balance to 100%					

Product pH is adjusted to 7.

20 In Examples 13-16 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

25 In Examples 17 and 18, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4 with substantially similar results.

Example 19
Granular Automatic Dishwashing Composition

<u>Component</u>	<u>A</u>	<u>B</u>	<u>C</u>
Citric Acid	15.0	-	-
Citrate	4.0	29.0	15.0
Acrylate/methacrylate copolymer	6.0	-	6.0
Acrylic acid maleic acid copolymer	-	3.7	-
Dry add carbonate	9.0	-	20.0
Alkali metal silicate	8.5	17.0	9.0
Paraffin	-	0.5	-
Benzotriazole	-	0.3	-
Termamyl 60T	1.5	1.5	1.0
Protease # 12 (4.6% prill)	1.6	1.6	1.6
Percarbonate (AvO)	1.5	-	-
Perborate monohydrate	-	0.3	1.5
Perborate tetrahydrate	-	0.9	-
Tetraacetylene diamine	3.8	4.4	-
Diethylene triamine penta methyl phosphonic acid (Mg salt)	0.13	0.13	0.13
Alkyl ethoxy sulphate - 3 times ethoxylated	3.0	-	-
Alkyl ethoxy propoxy nonionic surfactant	-	1.5	-
Suds suppressor	2.0	-	-
Olin SLF18 nonionic surfactant	-	-	2.0
Sulphate	Balance to 100%		

5 In Examples 19 A-C the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in Examples 19 A-C, any combination of the proteases useful in the present invention recited in Tables III, V and VI among 10 others, are substituted for Protease # 12 with substantially similar results.

15 3. Fabric cleaning compositions
 In another embodiment of the present invention, fabric cleaning compositions comprise one or more protease enzymes. As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for cleaning fabrics, including but not limited to, granular, liquid and bar forms.

a. Granular fabric cleaning compositions

The granular fabric cleaning compositions of the present invention contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1% by weight of active protease enzyme of the composition. In addition to one or more protease enzymes, the granular fabric cleaning compositions typically comprise at least one surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 20-23

Granular Fabric Cleaning Composition

		Example No.		
	Component	20	21	22
15	Protease # 12 (4% Prill)	0.10	0.20	0.03
	Protease # 4 (4% Prill)	-	-	0.02
	C13 linear alkyl benzene sulfonate	22.00	22.00	22.00
	Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00
20	Sodium carbonate	23.00	23.00	23.00
	Sodium silicate	14.00	14.00	14.00
	Zeolite	8.20	8.20	8.20
	Chelant (diethylenetriamine-pentaacetic acid)	0.40	0.40	0.40
25	Sodium sulfate	5.50	5.50	5.50
	Water	balance to 100%		

In Examples 20-21 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 22 and 23, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 24-27

Granular Fabric Cleaning Composition

Component	Example No.			
	24	25	26	27
5 Protease # 12 (4% Prill)	0.10	0.20	0.03	0.05
↳ Protease # 4 (4% Prill)	-	-	0.02	0.05
C ₁₂ alkyl benzene sulfonate	12.00	12.00	12.00	12.00
Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
2-butyl octanoic acid	4.00	4.00	4.00	4.00
10 C ₁₂ -C ₁₄ secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
Sodium citrate	5.00	5.00	5.00	5.00
Optical brightener	0.10	0.10	0.10	0.10
Sodium sulfate	17.00	17.00	17.00	17.00
15 Fillers, water, minors	balance to 100%			

In Examples 24 and 25 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

20 In Examples 26 and 27, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 28 and 29
Granular Fabric Cleaning Compositions

<u>Components</u>	<u>Example No.</u>	
	<u>28</u>	<u>29</u>
Linear alkyl benzene sulphonate	11.4	10.70
Tallow alkyl sulphate	1.80	2.40
C14-15 alkyl sulphate	3.00	3.10
C14-15 alcohol 7 times ethoxylated	4.00	4.00
Tallow alcohol 11 times ethoxylated	1.80	1.80
Dispersant	0.07	0.1
Silicone fluid	0.80	0.80
Trisodium citrate	14.00	15.00
Citric acid	3.00	2.50
Zeolite	32.50	32.10
Maleic acid acrylic acid copolymer	5.00	5.00
Diethylene triamine penta methylene phosphonic acid	1.00	0.20
Protease # 12 (4% Prill)	0.30	0.30
Lipase	0.36	0.40
Amylase	0.30	0.30
Sodium silicate	2.00	2.50
Sodium sulphate	3.50	5.20
Polyvinyl pyrrolidone	0.30	0.50
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.1	0.1
Minors	Up to 100	Up to 100

5

Examples 30 and 31
Granular Fabric Cleaning Compositions

<u>Components</u>	<u>Example No.</u>	
	<u>30</u>	<u>31</u>
Sodium linear C12 alkyl benzene-sulfonate	6.5	8.0
Sodium sulfate	15.0	18.0
Zeolite A	26.0	22.0
Sodium nitrilotriacetate	5.0	5.0
Polyvinyl pyrrolidone	0.5	0.7
Tetraacetylethylene diamine	3.0	3.0

Boric acid	4.0	-
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Protease # 12 (4% Prill)	0.4	0.4
Fillers (e.g., silicates; carbonates; perfumes; water)	Up to 100	Up to 100

Example 32Compact Granular Fabric Cleaning Composition

<u>Components</u>	<u>Weight %</u>
Alkyl Sulphate	8.0
Alkyl Ethoxy Sulphate	2.0
Mixture of C25 and C45 alcohol 3 and 7 times ethoxylated	6.0
Polyhydroxy fatty acid amide	2.5
Zeolite	17.0
Layered silicate/citrate	16.0
Carbonate	7.0
Maleic acid acrylic acid copolymer	5.0
Soil release polymer	0.4
Carboxymethyl cellulose	0.4
Poly (4-vinylpyridine) -N-oxide	0.1
Copolymer of vinylimidazole and vinylpyrrolidone	0.1
PEG2000	0.2
Protease # 12 (4% Prill)	0.5
Lipase	0.2
Cellulase	0.2
Tetracetylene diamine	6.0
Percarbonate	22.0
Ethylene diamine disuccinic acid	0.3
Suds suppressor	3.5
Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate	0,25
Disodium-4,4'-bis (2-sulfostyryl) biphenyl	0.05
Water, Perfume and Minors	Up to 100

Example 33Granular Fabric Cleaning Composition

<u>Component</u>	<u>Weight %</u>
Linear alkyl benzene sulphonate	7.6

C ₁₆ -C ₁₈ alkyl sulfate	1.3
C ₁₄₋₁₅ alcohol 7 times ethoxylated	4.0
Coco-alkyl-dimethyl hydroxyethyl ammonium chloride	1.4
Dispersant	0.07
Silicone fluid	0.8
↳ Trisodium citrate	5.0
Zeolite 4A	15.0
Maleic acid acrylic acid copolymer	4.0
Diethylene triamine penta methylene phosphonic acid	0.4
Perborate	15.0
Tetraacetylethylene diamine	5.0
Smectite clay	10.0
Poly (oxy ethylene) (MW 300,000)	0.3
Protease # 12 (4% Prill)	0.4
Lipase	0.2
Amylase	0.3
Cellulase	0.2
Sodium silicate	3.0
Sodium carbonate	10.0
Carboxymethyl cellulose	0.2
Brighteners	0.2
Water, perfume and minors	Up to 100

Example 34Granular Fabric Cleaning Composition

<u>Component</u>	<u>Weight %</u>
Linear alkyl benzene sulfonate	6.92
Tallow alkyl sulfate	2.05
C ₁₄₋₁₅ alcohol 7 times ethoxylated	4.4
C ₁₂₋₁₅ alkyl ethoxy sulfate - 3 times ethoxylated	0.16
Zeolite	20.2
Citrate	5.5
Carbonate	15.4
Silicate	3.0
Maleic acid acrylic acid copolymer	4.0
Carboxymethyl cellulase	0.31
Soil release polymer	0.30
Protease # 12 (4% Prill)	0.2

Lipase	0.36
Cellulase	0.13
Perborate tetrahydrate	11.64
Perborate monohydrate	8.7
Tetraacetylethylene diamine	5.0
Diethylene tramine penta methyl phosphonic acid	0.38
Magnesium sulfate	0.40
Brightener	0.19
Perfume, silicone, suds suppressors	0.85
Minors	Up to 100

In each of Examples 28-34 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 28-34, any 5 combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

b. Liquid fabric cleaning compositions

Liquid fabric cleaning compositions of the present invention comprise an 10 effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.001% to about 0.1%, by weight of active protease 15 enzyme of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a fatty acid, a water-soluble detergency builder and water.

The liquid fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 35-39
Liquid Fabric Cleaning Compositions

	Component	Example No.				
		35	36	37	38	39
5	Protease # 12	0.05	0.03	0.30	0.03	0.10
	Protease # 4	-	-	-	0.01	0.20
	C ₁₂ - C ₁₄ alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
	2-Butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
	Sodium citrate	1.00	1.00	1.00	1.00	1.00
10	C ₁₀ alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
	Monethanolamine	2.50	2.50	2.50	2.50	2.50
	Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

In Examples 35-37 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 38 and 39, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 40-41
Liquid Fabric Cleaning Compositions

	<u>Example No.</u>	
	<u>40</u>	<u>41</u>
5	C ₁₂ -14 alkenyl succinic acid	3.0 8.0
	Citric acid monohydrate	10.0 15.0
	Sodium C ₁₂ -15 alkyl sulphate	8.0 8.0
	Sodium sulfate of C ₁₂ -15 alcohol 2 times ethoxylated	- 3.0
	C ₁₂ -15 alcohol 7 times ethoxylated	- 8.0
10	C ₁₂ -15 alcohol 5 times ethoxylated	8.0 -
	Diethylene triamine penta (methylene phosphonic acid)	0.2 -
	Oleic acid	1.8 -
	Ethanol	4.0 4.0
	Propanediol	2.0 2.0
15	Protease # 12	0.2 0.2
	Polyvinyl pyrrolidone	1.0 2.0
	Suds suppressor	0.15 0.15
	NaOH	up to pH 7.5
	Perborate	0.5 1
20	Phenol sulphonate	0.1 0.2
	Peroxidase	0.4 0.1
	Waters and minors	up to 100 parts

In each of Examples 40 and 41 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 40 and 41, any combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted from Protease # 12 with substantially similar results.

30 c. Bar fabric cleaning compositions

Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

35 The bar fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 42-45
Bar Fabric Cleaning Compositions

	<u>Component</u>	<u>Example No.</u>			
		42	43	44	45
5	Protease # 12	0.3	-	0.1	0.02
	Protease # 4	-	-	0.4	0.03
	C ₁₂ -C ₁₆ alkyl sulfate, Na	20.0	20.0	20.0	20.00
	C ₁₂ -C ₁₄ N-methyl glucamide	5.0	5.0	5.0	5.00
	C ₁₁ -C ₁₃ alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
10	Sodium carbonate	25.0	25.0	25.0	25.00
	Sodium pyrophosphate	7.0	7.0	7.0	7.00
	Sodium tripolyphosphate	7.0	7.0	7.0	7.00
	Zeolite A (0.1-.10 μ)	5.0	5.0	5.0	5.00
	Carboxymethylcellulose	0.2	0.2	0.2	0.20
15	Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
	Coconut monethanolamide	5.0	5.0	5.0	5.00
	Brightener, perfume	0.2	0.2	0.2	0.20
	CaSO ₄	1.0	1.0	1.0	1.00
	MgSO ₄	1.0	1.0	1.0	1.00
20	Water	4.0	4.0	4.0	4.00
	<u>Filler*</u>	balance to 100%			

*Can be selected from convenient materials such as CaCO₃, talc, clay, silicates, and the like.

In Examples 42 and 43 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 44 and 45, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

B. Additional Cleaning Compositions

In addition to the hard surface cleaning, dishwashing and fabric cleaning compositions discussed above, one or more protease enzymes may be incorporated into a variety of other cleaning compositions where hydrolysis of an insoluble substrate is desired. Such additional cleaning compositions

include but are not limited to, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning compositions.

1. Oral cleaning compositions

In another embodiment of the present invention, a pharmaceutically-acceptable amount of one or more protease enzymes are included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, oral cleaning compositions of the present invention comprise from about 0.0001% to about 20% of one or more protease enzymes, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-acceptable" means that drugs, medicaments or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier components of the oral cleaning components of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The pharmaceutically-acceptable carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in U.S. Patent 5,096,700, Seibel, issued March 17, 1992; U.S. Patent 5,028,414, Sampathkumar, issued July 2, 1991; and U.S. Patent 5,028,415, Benedict, Bush and Sunberg, issued July 2, 1991; all of which are incorporated herein by reference.

The oral cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 46-49
Dentifrice Composition

	<u>Component</u>	<u>Example No.</u>			
		46	47	48	49
5	Protease # 12	2.000	3.500	1.500	2.000
	Sorbitol (70% aqueous solution)	35.000	35.000	35.000	35.000
	PEG-6*	1.000	1.000	1.000	1.000
	Silica dental abrasive**	20.000	20.000	20.000	20.000
	Sodium fluoride	0.243	0.243	0.243	0.243
10	Titanium dioxide	0.500	0.500	0.500	0.500
	Sodium saccharin	0.286	0.286	0.286	0.286
	Sodium alkyl sulfate (27.9% aqueous solution)	4.000	4.000	4.000	4.000
	Flavor	1.040	1.040	1.040	1.040
15	Carboxyvinyl Polymer***	0.300	0.300	0.300	0.300
	Carrageenan****	0.800	0.800	0.800	0.800
	Water	balance to 100%			

*PEG-6 = Polyethylene glycol having a molecular weight of 600.

**Precipitated silica identified as Zeodent 119 offered by J.M. Huber.

20 ***Carbopol offered by B.F. Goodrich Chemical Company.

****Iota Carrageenan offered by Hercules Chemical Company.

In Examples 46-49 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with 25 substantially similar results. Also in Examples 46-49, any combination of the protease enzymes useful in the present invention recited in Tables III, V, VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 50-53
Mouthwash Composition

		Example No.			
	Component	50	51	52	53
5	Protease # 12	3.00	7.50	1.00	5.00
	SDA 40 Alcohol	8.00	8.00	8.00	8.00
	Flavor	0.08	0.08	0.08	0.08
	Emulsifier	0.08	0.08	0.08	0.08
	Sodium Fluoride	0.05	0.05	0.05	0.05
10	Glycerin	10.00	10.00	10.00	10.00
	Sweetener	0.02	0.02	0.02	0.02
	Benzoic acid	0.05	0.05	0.05	0.05
	Sodium hydroxide	0.20	0.20	0.20	0.20
	Dye	0.04	0.04	0.04	0.04
15	Water	balance to 100%			

In Examples 50-53 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 50-53, any combination of the 20 protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 54-57
Lozenge Composition

		Example No.			
	Component	54	55	56	57
	Protease # 12	0.01	0.03	0.10	0.02
	Sorbitol	17.50	17.50	17.50	17.50
	Mannitol	17.50	17.50	17.50	17.50
30	Starch	13.60	13.60	13.60	13.60
	Sweetener	1.20	1.20	1.20	1.20
	Flavor	11.70	11.70	11.70	11.70
	Color	0.10	0.10	0.10	0.10
	Com Syrup	balance to 100%			

35 In Examples 54-57 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with

substantially similar results. Also in Examples 54-57, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

5

Examples 58-61

Chewing Gum Composition

		Example No.		
	Component	58	59	60
	Protease # 12	0.03	0.02	0.10
10	Sorbitol crystals	38.44	38.40	38.40
	Paloja-T gum base*	20.00	20.00	20.00
	Sorbitol (70% aqueous solution)	22.00	22.00	22.00
	Mannitol	10.00	10.00	10.00
	Glycerine	7.56	7.56	7.56
15	<u>Flavor</u>	1.00	1.00	1.00

*Supplied by L.A. Dreyfus Company.

In Examples 58-61 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 58-61, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

25

2. Denture cleaning compositions

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more protease enzymes. Such denture cleaning compositions comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 50% of one or more protease enzymes, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see for example U.S. Patent 5,055,305, Young, incorporated herein by reference), and are generally appropriate for incorporation of one or more protease enzymes for removing proteinaceous stains from dentures.

The denture cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 62-65

Two-layer Effervescent Denture Cleansing Tablet

5	Component	Example No.			
		62	63	64	65
<u>Acidic Layer</u>					
	Protease # 12	1.0	1.5	0.01	0.05
	Tartaric acid	24.0	24.0	24.00	24.00
10	Sodium carbonate	4.0	4.0	4.00	4.00
	Sulphamic acid	10.0	10.0	10.00	10.00
	PEG 20,000	4.0	4.0	4.00	4.00
	Sodium bicarbonate	24.5	24.5	24.50	24.50
	Potassium persulfate	15.0	15.0	15.00	15.00
15	Sodium acid pyrophosphate	7.0	7.0	7.00	7.00
	Pyrogenic silica	2.0	2.0	2.00	2.00
	Tetracetylethylene diamine	7.0	7.0	7.00	7.00
	Ricinoleylsulfosuccinate	0.5	0.5	0.50	0.50
	Flavor	1.0	1.0	1.00	1.00
20	<u>Alkaline Layer</u>				
	Sodium perborate monohydrate	32.0	32.0	32.00	32.00
	Sodium bicarbonate	19.0	19.0	19.00	19.00
	EDTA	3.0	3.0	3.00	3.00
	Sodium tripolyphosphate	12.0	12.0	12.00	12.00
25	PEG 20,000	2.0	2.0	2.00	2.00
	Potassium persulfate	26.0	26.0	26.00	26.00
	Sodium carbonate	2.0	2.0	2.00	2.00
	Pyrogenic silica	2.0	2.0	2.00	2.00
	Dye/Flavor	2.0	2.0	2.00	2.00

30 In Examples 62-65 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 62-65, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

3. Personal Cleansing Compositions

In another embodiment of the present invention, personal cleaning compositions for cleaning the skin (in liquid and bar form) comprise one or more of the protease enzymes. Such compositions typically comprise from about 0.001% to about 5% protease enzyme, preferably from about 0.005% to 5 about 2%, and most preferably from about 0.01% to about 0.8% by weight of the composition. Preferred personal cleansing compositions into which can be included protease enzymes as described herein are taught in U.S. Patent Applications SN 08/121,623 and SN 08/121,624, both by Visscher et al., filed September 14, 1993, the disclosures of which are incorporated herein by 10 reference in their entirety. Such compositions are illustrated by the following examples.

Example 66

Liquid Personal Cleansing Compositions Containing Soap

<u>Component</u>	<u>Weight %</u>
15 Soap (K or Na)	15.00
30% Laurate	
30% Myristate	
25% Palmitate	
15% Stearate	
20 Fatty acids (above ratios)	4.50
Na Lauryl Sarcosinate	6.00
Sodium Laureth-3 Sulfate	0.66
Cocamidopropylbetaine	1.33
Glycerine	15.00
25 Propylene glycol	9.00
Polyquaternium 10	0.80
Ethylene glycol distearate (EDTA)	1.50
Propylparaben	0.10
Methylparaben	0.20
30 Protease # 12	0.10
KOH or NaOH	If necessary to adjust pH
Calcium sulfate	3
Acetic acid	3
Water	Balance to 100

Example 67

Personal Cleansing Bar Composition

<u>Component</u>	<u>Weight %</u>
------------------	-----------------

	Sodium Cocoyl Isethionate	47.20
	Sodium Cetearyl Sulfat	9.14
	Paraffin	9.05
	Sodium Soap (in situ)	3.67
5	Sodium Isethionate	5.51
	Sodium Chloride	0.45
	Titanium Dioxide	0.4
	Trisodium EDTA	0.1
	Trisodium Etidronate	0.1
10	Perfume	1.20
	Na ₂ SO ₄	0.87
	Protease # 12	0.10
	Water/Minors	Balance to 100

In Examples 66-67 the Proteases #'s 1-11 and 13-25 recited in Table III, 15 among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 66-67, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar 20 results.

Example 68

Wash Performance Test

The wash performance of the variants useful in the present invention compositions is evaluated by measuring the removal of stain from EMPA 116 25 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030).

Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, are placed in each pot of a Model 7243S Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness 30 (Ca⁺⁺:Mg⁺⁺::3:1::w:w), 7 g of detergent, and enzyme as appropriate. The detergent base is WFK1 detergent from wfk - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda Ash	10%
Linear alkylbenzenesulfonate	8.8%

Alcohol ethoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO ₂ :Na ₂ O::3.3:1)	3%

To this base detergent, the following additions are made:

Component	% of Final Formulation
Sodium perborate monohydrate	13%
Copolymer (Sokalan CP5)	4%
TAED (Mykon ATC Green)	3%
Enzyme	0.5%
Brightener (Tinopal AMS-GX)	0.2%

5 Sodium perborate monohydrate is obtained from Degussa Corporation, Ridgefield-Park, NJ 07660. Sokalan CP5 is obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetyl ethylenediamine) is obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX is obtained from Ciba-Geigy Corporation, 10 Greensboro, NC 27419.

10 Six EMPA 116 swatches are washed in detergent with enzyme for 30 min at 60°C and are subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes are added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses. Swatches are dried and 15 pressed, and the reflectance from the swatches is measured using the L value on the L*a*b* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. latus* (GG36) protease and is calculated by dividing the amount of *B. latus* (GG36) protease by the amount of variant protease that is 20 needed to provide the same stain removal performance X 100. The data are shown in Table VII.

Table VII

Enzyme	Wash Performance
<i>B. latus</i> subtilisin	100
N76D	310
N76D/S103A	230
N76D/V104I	130
N76D/V107V	160
N76D/S99D/S101R	370
N76D/S99D/S103A	290
N76D/S101R/S103A	130
N76D/S101R/V104I	300
N76D/S103A/V104I	320
N76D/S103G/V104I	160
N76D/S103A/V104F	210
N76D/S103A/V104N	110

N76D/S103AAV104T	170
N76D/V104I/I107V	210
N76D/S99D/S101R/S103A	220
N76D/S99D/S101R/V104I	140
N76D/S101G/S103A/V104I	170
N76D/S101R/S103AAV104I	150
N76D/S103AAV104I/S105A	170
N76D/S103AAV104T/I107A	120
N76D/S103AAV104T/I107L	110
N76D/S103AAV104I/L126F	110
N76D/S103AAV104I/S128G	280
N76D/S103AAV104I/L135I	160
N76D/S103AAV104I/L135V	160
N76D/S103AAV104I/D197E	170
N76D/S103AAV104I/N204A	160
N76D/S103AAV104I/N204G	150
N76D/S103AAV104I/P210I	470
N76D/S103AAV104I/M222A	100
N76D/S103AAV104I/T260P	280
N76D/S103AAV104I/S265N	190

Example 69Protease Stability in a Liquid Detergent Formulation

A comparison of protease stability toward inactivation in a liquid detergent formulation is made for *Bacillus lenthus* subtilisin and it's variant 5 enzyme N76D/S103AAV104I according to the procedure outlined herein. The detergent formulation for the study is a commercially purchased bottle of Tide Ultra liquid laundry detergent made in the USA by The Procter & Gamble Company. Heat treatment of the detergent formulation is necessary to inactivate *in-situ* protease. This is accomplished by incubating the detergent at 10 96°C for a period of 4.5 hours. Concentrated preparations of the *B. lenthus* subtilisin and N76D/S103AAV104I variant, in the range of 20 grams/liter enzyme, are then added to the heat-treated Tide Ultra at room-temperature to a final concentration of 0.3 grams/liter enzyme in the detergent formulation. The heat-treated detergent with protease added is then incubated in a water bath 15 thermostatted at 50°C. Aliquots are removed from the incubation tubes at 0, 24, 46, 76, and 112 hour time intervals and assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate suc-Ala-Ala-Pro-phe-p-nitroanilide dissolved in 0.1M tris-HCL buffer, pH 8.6, and thermostatted at 25°C. The initial linear reaction velocity is followed 20 spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410nm as a function of time. As shown in Fig. 10, the N76D/S103AAV104I variant is observed to have significantly greater stability towards inactivation than the native *B. lenthus* enzyme. Estimated half-lives for inactivation in the Tide Ultra detergent formulation for the two enzymes, under

the specified test conditions, are 45 hours for *B. lenthus* subtilisin and 125 hours for the N76D/S103A/V104I variant.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and 5 modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.

SEQUENCE LISTING

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Rubingh, Donn N.

(ii) TITLE OF INVENTION: Protease-Containing Cleaning Compositions

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: The Procter & Gamble Company
(B) STREET: 11810 East River Road
(C) CITY: Cincinnati
(D) STATE: OH
(E) COUNTRY: USA
(F) ZIP: 45253-8707

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patentin Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 13-OCT-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 32,323
(C) REFERENCE/DOCKET NUMBER: 5040R

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (513) 627-2885
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGCTGCAA CTCGTTAAA

19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGCTCTAG ACAATTAG 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATTAGGGG CGGACGGTCC AGGCGCCATC AGCTCGATT 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGGTTCCG TCTCGAGCGT TGCCCAAGGA TTG 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACGTTGCTA CCTTGAGTTT AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1497 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCTACTAA AATATTATTC CATACTATAC AATTAAATACA CAGAATAATC TGTCTATGG 60

TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA 120

GTTTGCTGTT	TGCTTTAGCG	TTAATCTTA	CGATGGCGTT	CGGCAGCACA	TCCTCTGCC	180	
AGGCAGGCAAG	GAATCAAAAC	GGGGAAAAGA	AATATATTGT	CGGGTTAAA	CAGACAATGA	240	
GCACGGATGAG	CGCCGCTAAG	AAGAAAGATG	TCATTTCTGA	AAAAGGCGGG	AAAGTGCAAA	300	
ACCAATTCAA	ATATGTAGAC	GCAGCTTCAG	TCACATTAAA	CGAAAAAGCT	GTAAAAGAAI	360	
TGAAAAAAGA	CCCGAGCGTC	GCTTACGTTG	AAGAAGATCA	CGTAGCACAT	GGTAGCGCGC	420	
AGTCCGTGCC	TTACGGCGTA	TCACAAATTAA	AAGCCCCTGC	TCTGCACTCT	CAAGGCTACA	480	
CTGGATCAAA	TGTTAAAGTA	GCGGTTATCG	ACAGCGGTAT	CGATTCTCT	CATCCTGATT	540	
TAAAGGTAGC	AAGCGGAGCC	AGCATGGTTC	CTTCTGA	AAC	AAATCCTTC	CAAGACAAACA	600
ACTCTCACGG	AACTCACGTT	GCCGGCACAG	TTGCGGCTCT	TAATAACTCA	ATCGGTGTAT	660	
TAGGCCTTGC	GCCAAGCGCA	TCACTTTACG	CTGTAAAAGT	TCTCGTGCT	GACGGTTCCG	720	
GCCAATACAG	CTGGATCATT	AACGGATCG	AGTGGCGAT	CGCAAACAAT	ATGGACGTTA	780	
TTAACATGAG	CCTCGGGCGGA	CCTTCTGGTT	CTGCTGCTTT	AAAAGCGGCA	GTTGATAAAG	840	
CCGTTGCATC	CGGCCTCGTA	GTCGTTGCGG	CAGCCGGTAA	CGAAGGCACT	TCCGGCAGCT	900	
CAAGCACAGT	GGGCTACCC	GGTAAATACC	CTTCTGTAT	TGCACTAGGC	GCTGTTGACA	960	
GCAGCAACCA	AAGAGCATCT	TTCTCAAGCG	TAGGACCTGA	GCTTGATGTC	ATGGCACCTG	1020	
GGGTATCTAT	CCAAAGCAGC	CTTCCTGGAA	ACAAATACGG	GGCGTACAAC	GGTACGTCAA	1080	
TGGCATCTCC	GCACGTTGCC	GGAGCGGCTG	CTTGTATTCT	TTCTAAGCAC	CCGAACTGGA	1140	
CAAAACACTCA	AGTCCGCAGC	AGTTTAGAAA	ACACCACTAC	AAAATTTGGT	GATTCTTTGT	1200	
ACTATGGAAA	AGGGCTGATC	AACGTACAAG	CGGCAGCTCA	GTAAAACATA	AAAAACCGGC	1260	
CTTGGCCCCG	CGGGTTTTTT	ATTATTTTC	TTCCCTCGCA	TGTTCAATCC	GCTCCATAAT	1320	
CGACGGATGG	CTCCCTCTGA	AAATTTAAC	GAGAACGGC	GGGTTGACCC	GGTCAGTCC	1380	
CGTAACGGCC	AACTCCGAA	ACGTCTCAAT	CGCCGTTCC	CGGTTCCGG	TCAGCTCAAT	1440	
CCCATAACCGG	TCGGCGGCGT	TTTCCTGATA	CCGGGAGACG	GCATTCGAA	TCGGATC	1497	

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu
1								5			10			15	

Mis	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp
								20			25			30	

Ser	Gly	Ile	Asp	Ser	Ser	Mis	Pro	Asp	Leu	Lys	Val	Ala	Gly	Gly	Ala
						35		40			45				

60

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
 50 55 60

Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80

Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95

Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110

Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125

Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
 130 135 140

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
 145 150 155 160

Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
 165 170 175

Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
 180 185 190

Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
 210 215 220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
 225 230 235 240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Lys
 245 250 255

Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270

Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu
 1 5 10 15

His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
 20 25 30

Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala
 35 40 45

Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His
 50 55 60

Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80

Val Leu Gly Val Ser Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125
 Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser
 130 135 140
 Ser Gly Ile Val Val Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
 145 150 155 160
 Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala
 165 170 175
 Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala
 180 185 190
 Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr
 210 215 220
 Pro His Val Ala Gly Ala Ala Leu Ile Leu Ser Lys His Pro Thr
 225 230 235 240
 Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr
 245 250 255
 Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 274 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val
 1 5 10 15
 Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala
 35 40 45
 Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly
 50 55 60
 Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val
 65 70 75 80
 Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys Val Leu Asn
 85 90 95

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Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly Ile Glu Trp
 100 105 110
 Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu Gly Gly Ala
 115 120 125
 Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala Tyr Ala Arg
 130 135 140
 Gly Val Val Val Val Ala Ala Ala Gly Asn Ser Gly Asn Ser Gly Ser
 145 150 155 160
 Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val Ile Ala Val
 165 170 175
 Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser Ser Val Gly
 180 185 190
 Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr Ser Thr Tyr
 195 200 205
 Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met Ala Ser Pro
 210 215 220
 His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu
 225 230 235 240
 Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu
 245 250 255
 Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala
 260 265 270
 Ala Gln

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala
 1 5 10 15
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
 35 40 45
 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
 50 55 60
 His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
 65 70 75 80
 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
 85 90 95
 Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
 100 105 110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
 115 120 125
 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
 130 135 140
 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
 145 150 155 160
 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
 165 170 175
 Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
 180 185 190
 Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
 195 200 205
 Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
 210 215 220
 Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
 225 230 235 240
 Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
 245 250 255
 Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
 260 265

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1140 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGAAGAAC CGTTGGGAA AATTGTCGCA ACCACCGCAC TACTCATTTC	TGTTGCTTT	60
AGTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT	TGGCTTTAAT	120
GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA	GGTCGCCATT	180
CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTGAAAC	GATTCCCTGTT	240
TTATCCGTTG AGTTAACGCC AGAAGATGTG GACGGCGCTTG AACTCGATCC	AGCGATTTC	300
TATATTGAAG AGGATGCCAGA AGTAACGACA ATGGCCCAAT CAGTCCCCATG	GGGAATTAGC	360
CCTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT	AAAAGTTGCT	420
GTCCCTCGATA CAGGTATTTC CACTCATCCA GACTTAAATA TTCTGTGGTGG	CGCTAGCTT	480
GTACCAAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT	GGCCGGGACG	540
ATTGCTGCTT TAAACAATTTC GATGGCGTT CTTGGCGTAG CGCCGAGCGC	GGAACTATAC	600
GCTGTTAAAG TATTAAGGGGC GAGCGGTCA GGTCGGTCA GCTCGATTGC	CCAAGGATTG	660
GAATGGGCAG GGAACAAATGG CATGCCACGTT GCTAATTGTA GTTGTAGGAAG	CCCTTCGCCA	720
AGTGCCACAC TTGAGCAAGC TGTTAATAGC CGGACTTCTA GAGGCAGTCT	TGTTGTAGCG	780
GCATCTGGGA ATTCAAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC	GAACGCAATG	840

GCAGTCGGAG CTACTGACCA AAACAACAAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG	900
CTTGACATTG TCGCACCAAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC	960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTAAA	1020
CAAAGAACC CATCTTGGTC CAATGTACAA ATCCGAATC ATCTAAAGAA TACGGCAACG	1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC	1140

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1140 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAAGAAAC CGTTGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTT	60
AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT	120
GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT	180
CTCTCTGAGG AAGAGGAAGT CGAAATGAA TTGCTTCATG AATTGAAAC GATTCTGTT	240
TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCCCTG AACTCGATCC AGCGATTCT	300
TATATTGAAG AGGATGCGA AGTAACCGACA ATGGCCAAAT CAGTGCCATG GGGAAATTGC	360
CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT	420
GTCCTCGATA CAGGTATTTCA CACTCATCCA GACTTAAATA TTCTGGTGG CGCTAGCTT	480
GTACCAAGGG ACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG	540
ATTGCTGTT TAGACAACTC GATTGGCGTT CTTGGCTAG CGCCGAGCGC GGAACATAC	600
GCTGTTAAAG TATTAGGGC GAGCGGTTCA GGCGCCATCA GCTCGATTGC CCAAGGATTG	660
GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTGA GTTAGGAAG CCCTTCGCCA	720
AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCCTTCT TGTGTAGCG	780
GCATCTGGGA ATTCAAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG	840
GCAGTCGGAG CTACTGACCA AAACAACAAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG	900
CTTGACATTG TCGCACCAAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC	960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTAAA	1020
CAAAGAACC CATCTTGGTC CAATGTACAA ATCCGAATC ATCTAAAGAA TACGGCAACG	1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC	1140

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATGCCAGCC ACAACGGTAC TTTCGATGGCT

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T

31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT

33

What is claimed is:**1. A cleaning composition comprising:**

(a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin; and

(b) one or more cleaning composition materials compatable with the protease enzyme.

2. The cleaning compositions according to Claim 1 wherein the cleaning composition materials are selected from the group consisting of surfactants, solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, builders, bleaching agents, dyes, perfumes, and mixtures thereof.**3. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least about 1% surfactant by weight of the composition, said surfactant comprising materials selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof.****4. The cleaning composition according to Claim 3 further comprising at least about 5% builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.**

5. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one bleaching agent.
6. The cleaning compositions according to Claim 5 wherein the bleaching agent is selected from the group consisting of percarbonates, perborates, and mixtures thereof, and optionally further comprising at least one bleach activator.
7. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases and mixtures thereof.
8. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one fabric softener.
9. The cleaning compositions according to Claim 1 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of substitutions is made at the positions equivalent to 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 5 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/99/101/103/104/123, 76/103/104/126, 76/103/104/135, 76/103/104/197, 76/103/104/222, 10 76/103/104/260, 76/103/104/265, 76/103/104/126/265, 27/76/104/123/274, 27/76/104/109/123/274, 27/76/104/123/218/274, 27/76/104/123, 27/76/104/107/123, 27/76/104/109/123, 27/76/104/109/123/218/274, 27/76/104/123/197, 27/76/104/123/204, 27/76/104/123/206, 27/76/104/123/216, 27/76/104/123/218, 27/76/104/123/260, 15 27/76/104/123/195/197, 27/76/104/123/195/218, 27/76/104/123/197/218, 27/76/104/123/204/218, 27/76/104/123/206/218, 27/76/104/123/218/260, 27/76/104/123/195/197/218, 76/103/104/217, 76/103/104/156, 76/103/104/166, 76/103/104/105, 76/101/103/104, 76/103/104/128, 76/103/104/210, 76/103/104/107, 76/103/104/204, 76/217, 76/103/104/156/166 and 20 76/103/104/128.

10. The cleaning composition according to Claim 9 wherein the protease enzyme is a subtilisin variant selected from the group consisting of 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 5 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/99/101/103/104/123, 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222.

11. A fabric cleaning composition comprising:

(a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; 5 N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;

10 N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S;

N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;

15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N;

N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A;

N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F;

N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N and

N76D/S103A/V104I/M222A and mixtures thereof; and

20 (b) one or more cleaning composition materials compatible with the protease enzyme comprising at least about 5% surfactant and at least about 5% builder, by weight of the composition.

12. The fabric cleaning composition according to Claim 11 further comprising cleaning composition materials selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds

boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

13. The fabric cleaning composition according to Claim 11 further comprising at least one bleaching agent.

14. The fabric cleaning composition according to Claim 11 further comprising at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases, and mixtures thereof.

15. The fabric cleaning composition according to Claim 11 in the form of a liquid, granule or bar.

16. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a subtilisin variant selected from N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V, N76D/S101R/S103A/V104I, N76D/S99D/S101R/S103A/V104I, N76D/S101R/V104I, and mixtures thereof.

5 17. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a *Bacillus* subtilisin variant selected from N76D/V104I, N76D/S103A/V104I, and mixtures thereof.

18. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin;

(b) at least about 5% surfactant;

5 (c) at least about 5% builder; and

(d) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

10 19. The fabric cleaning composition according to Claim 18 wherein the surfactant is selected from the group consisting of alkyl benzene sulfonates,

primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, 5 nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof; and wherein further the builder is selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

20. The fabric cleaning composition according to Claim 19 further comprising one or more cleaning composition materials selected from the group consisting of bleaching agents, fabric softeners, and enzymes.

21. The fabric cleaning composition according to Claim 18 in the form of a concentrated granular fabric cleaning composition comprising at least about 30% surfactant.

22. A dishwashing composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein 5 the protease enzyme is a subtilisin variant selected from N76D/S99D;

N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S;

N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I;

N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I;

N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;

10 N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;

N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I;

N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S;

N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S;

N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;

15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N;

N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A;

N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F;

N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and

N76D/S103A/V104I/M222A and mixtures thereof;

20 (b) from about 0.1% to about 10% surfactant; and

(c) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, dispersing agents, suds suppressors, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

23. The dishwashing composition according to Claim 22 wherein the protease enzyme is an N76D/S103A/V104I subtilisin variant derived from *Bacillus latus* subtilisin.

24. A personal cleansing composition comprising:

(a) from about 0.001% to about 5% by weight of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin;

(b) from about 0.01% to about 95% by weight of a surfactant system; and

(c) optionally, from about 0.05% to about 50% of an enzyme stabilizer.

25. The composition of Claim 24 wherein the protease enzyme is at a level of from about 0.001% to about 2% by weight.

26. The composition of Claim 25 wherein the protease enzyme is at a level of from about 0.01% to about 0.8% by weight.

27. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of anionic carboxylates, amine oxides, alkyl glucosides, glucose amides, alkyl sulfates, alkyl ether sulfates, acyl isethionates, alkyl sulfosuccinates, alkyl phosphate esters, ethoxylated phosphate esters, alkyl glyceryl ether sulfonates, or mixtures thereof.

28. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of soaps, acylglutamates, alkyl

sarcosinat s, lauramin oxides, cocamine oxides, cocamidopropylamine oxides, decylglucosides, lauryl sulfates, laureth sulfates, C₁₂-C₁₈ acyl isethionates, or mixtures thereof.

29. The composition of Claim 28 wherein the surfactant is soap at a level of at least about 2% by weight of the composition.

30. The composition of Claim 29 wherein the soap is at a level of at least about 10% by weight of the composition.

31. The composition of Claim 30 wherein the soap is at a level of at least about 25% by weight of the composition.

32. The composition of Claim 28 wherein the ratio of soap to protease enzyme is from about 2,000:1 to about 8:1.

33. The composition of Claim 32 wherein the ratio of soap to protease enzyme is from about 400:1 to about 40:1.

34. The composition of Claim 28 wherein the protease enzyme is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D;

5 N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;

10 N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I;

N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;

15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and N76D/S103A/V104I/M222A; and mixtures thereof.

35. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lenthus* subtilisin.

36. A method for cleaning dishes, said method comprising contacting a dish in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lenthus* subtilisin.

37. A method for personal cleansing, said method comprising contacting the part of the human or lower animal body in need of cleaning with a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the

5 protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N and N76D/S103A/V104I/M222A; and mixtures thereof.

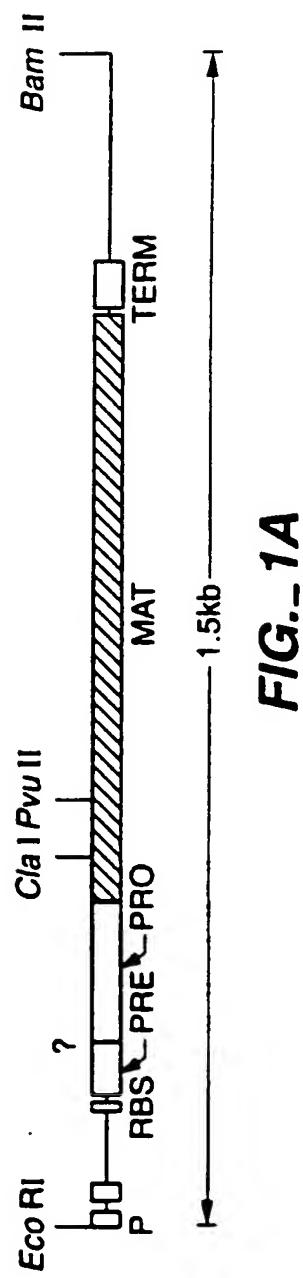


FIG. 1A

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1	<u>GGCTCACTAAATATTCCATACATACAAATTAAACACAGAAATAATC</u>		<u>GCTATGGTTATTCTGCAAATGAAAAAAGGAGGGATAAAGA</u>	GTG																						
99	Arg	Gly	Lys	Lys	Val	Ile	Ser	Leu	Leu	Phe	Ala	Leu	Ala	Ile	Phe	Thr	Met	Ala	Phe	Gly	Ser	Thr	Ser			
	AGA	GGC	AAA	AAA	GTA	TGG	ATC	AGT	TTC	CIG	TTT	GCT	TTA	GCG	TTA	ATC	TTT	ACG	ATG	GCG	TTC	GGC	AGC	ACA	TCC	
174	Ser	Ala	Gln	Ala	Ala	Gly	Lys	Ser	Asn	Gly	Glu	Lys	Lys	Tyr	Ile	Val	Gly	Phe	Lys	Gln	Thr	Met	Ser	Thr	Met	
	ICL	GCC	CAG	GCG	GCA	GGG	AAA	TCA	AAC	GCC	GGG	AAA	TCA	MAG	AAA	TAT	TAT	GTC	GGG	TTT	AAA	CAG	ACA	ATG	ACG	ATG
249	Ser	Ala	Ala	Lys	Lys	Lys	Lys	Asp	Val	Ile	Ser	Glu	Lys	Gly	Gly	Lys	Gln	Lys	Gln	Thr	Met	Ser	Thr	Met		
	AGC	GCC	GCT	AAG	AAG	AAG	AAG	GAT	GTC	ATT	TCT	GAA	AAA	GGC	GGG	AAA	GTG	CAA	AAG	CAA	TTC	AAA	TAT	GTA	GAC	GCA
324	Ala	Ser	Ala	Thr	Leu	Asn	Glu	Lys	Ala	Val	Lys	Glu	Leu	Lys	Lys	Asp	Pro	Ser	Val	Ala	Ile	Val	Val	Asp	Ala	
	GCT	TCA	GCT	ACA	TTA	AAC	GAA	AAA	GCA	TAA	GAA	TGA	AAA	TTC	AAA	AAA	GAC	CCG	AGC	GTC	GCT	TAC	GAA	GAA	GAT	
399	His	Val	Ala	His	Ala	Tyr	Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu	His	Ser	Gln	
	CAC	GTA	GCA	CAT	GCG	TAC	GCG	CAG	TCC	GRG	CCT	TAC	GGC	GTA	TCA	CAA	ATT	AAA	GCC	CCT	GCT	CTG	CAC	TCT	CAA	
474	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp	Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asp	Leu	Lys	Val	
	GGC	TAC	ACT	GGA	TCA	AAT	GTT	AAA	GTA	GGG	GTT	ATC	GAC	AGC	GGT	ATC	GAT	TCT	TCT	CAT	CCT	GAT	TTA	AAG	GTA	

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FIG.-1B-1

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FIG. - 1B - 2

1149	Gln	Val	Arg	Ser	Ser	250	Gln	Leu	Glu	Asn	Thr	Thr	Lys	Leu	Gly	Asp	Ser	Phe	Tyr	Gly	Lys	Leu	Ile	Asn				
	CAA	GTC	GGC	AGC	AGT	260	Gln	Glu	Glu	AAC	ACC	ACT	ACA	AAA	CTT	GGT	GAT	TCT	TTC	TAC	TAT	GGA	AAA	GGG	CTG	ATC	AAC	
270	Val	Gln	Ala	Ala	Ala	275	Val	Gln	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala								
1224	GTA	CAG	GCG	GCA	GCT	280	1224	GTA	CAG	GCG	GCA	GCT	CAG	TAA	<u>ACAT</u> <u>AAA</u> <u>ACCGG</u> <u>CCCT</u> <u>GGCT</u> <u>TTAA</u>	TERM												

1316 ATAACTGACGGAAAGGCTCCCTCTGAAAAATTAAACGAGAACGGGGTTGACCCGGCTCAGICCCGTAACGGCCAAAGTCCCTGAAACGTCTCAATCGCTCCG

1416 CTTCCGGTTCGGTCAGCTCAATGCCGTAACGGTGGCGGTTTCTGATACCGGAGACGGCATTCGTAATCGGATC

FIG.-1B - 3

FIG.-1B - 1

FIG.-1B - 2

FIG.-1B - 3

FIG.-1B

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CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

1	10	20
A Q S V P . G	A P A . H . . G	
21	30	40
. T G S . V K V A V . D . G	H P	
41	50	60
D L . . . G G A S . V P	Q D	
61	70	80
. N . H G T H V A G T . A A L N N S I G		
81	90	100
V L G V A P S A . L Y A V K V L G A . G		
101	110	120
S G . . S . L . . G . E W A . N		
121	130	140
V . N . S L G . P S . S A . .		
141	150	160
. G V . V V A A . G N . G . . .		
161	170	180
. Y P . . Y A V G A .		
181	190	200
D . . N . . A S F S . . G . . L D . . A		
201	210	220
P G V . . Q S T . P G . . Y . . . N G T		
221	230	240
S M A . P H V A G A A A L . . . K . . .		
241	250	260
W . . . Q . R . . L . N T . . . L G . .		
261	270	
. . Y G . G L . N . . A A . .		

FIG._2
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COMPARISON OF SUBTILISIN SEQUENCES FROM:

B. amyloliquefaciens

B. subtilis

B. licheniformis

B. lentus

01	A Q S V P Y G V S	10	Q I K A P A L H S	20	Q G Y T G S N V K V A V I D S S G I D S S H P
A Q S V P Y G I S Q	I K A P A L H S Q G Y T G S N V K V A V I D S S G I D S S H P	30	Q A Q G F K G A N V K V A V L D T G I Q A S H P		
A Q T V P Y G I P L	I K A D K V Q A Q G F K G A N V K V A V L D T G I S T * H P	40	R G L T G S G V K V A V L D T G I S T * H P		
A Q S V P W G I S R	V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P	50			
D L R V A G G A S M V P S B T N P P Q D N N S H G T H V A G T V A A L N N S I G	60				
D D L N V R G G A S P V P S B T N P Y Q D G S S H G T H V A G T I A A L N N S I G	70				
D L N V V G G A S P V A G B A Y N * T D G N G H G T H V A G T V A A L D N T T G	80				
D L N I R G G A S P V P G B * P S T Q D G N G H G T H V A G T I A A L N N S I G	90				
V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D	100				
V L G V S P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I S N N M D	110				
V L G V A P S V S L Y A V K V L G A S G S V S S I A Q G L E W A G N N G M H	120				
V L G V A P S A E L Y A V K V L G A S G S V S S I A Q G L E W A G N N G M H	130				
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V A A A G N E G T S G	140				
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A G N E G S S G	150				
V I N M S L G G A S G S T A M K Q A V D N A Y A R G V V V A A A G N S G N S G					
V A N L S L G S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S					

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FIG._3A

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161 S S S T V G Y P G K Y P S V I A V G A V D S S N Q R A S F S V G P E L D V M A
162 S T S T V C Y P A K Y P S T I A V G A V N S S N Q R A S F S S A G S E L D V M A
163 S T N T I G Y P A K Y D S V I A V G A V D S N R A S F S S V G A E L E V M A
164 * * * * I S Y P A R Y A N A M A V G A T D Q N N N R A S F S Q Y G A G L D I V A
165 170 175 180 185 190 195

201 P G V S I Q S T L P G N K Y G A Y N C T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T L P G C T Y G A Y N C T S M A T P H V A G A A A L I L S K H P T
P G V S I Q S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S
210 P G V S I Q S T L P G N K Y G A Y N C T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T L P G C T Y G A Y N C T S M A T P H V A G A A A L I L S K H P T
P G V S I Q S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S
220 P G V S I Q S T L P G N K Y G A Y N C T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T L P G C T Y G A Y N C T S M A T P H V A G A A A L I L S K H P T
P G V S I Q S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S
230 P G V S I Q S T L P G N K Y G A Y N C T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T L P G C T Y G A Y N C T S M A T P H V A G A A A L I L S K H P T
P G V S I Q S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S

241 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
242 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
243 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
244 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
245 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
246 250 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
247 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
248 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
249 254 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
250 258 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
251 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
252 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
253 257 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
254 258 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
255 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
256 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
257 261 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
258 262 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
259 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
260 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
261 265 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
262 266 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
263 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
264 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
265 269 W T N T Q V R S S L B N T T K L C D S F Y Y G K G L I N V Q A A Q
266 270 W T N A Q V R D R L B S T A T Y L G N S F Y Y G K G L I N V Q A A Q
267 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A Q
268 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R

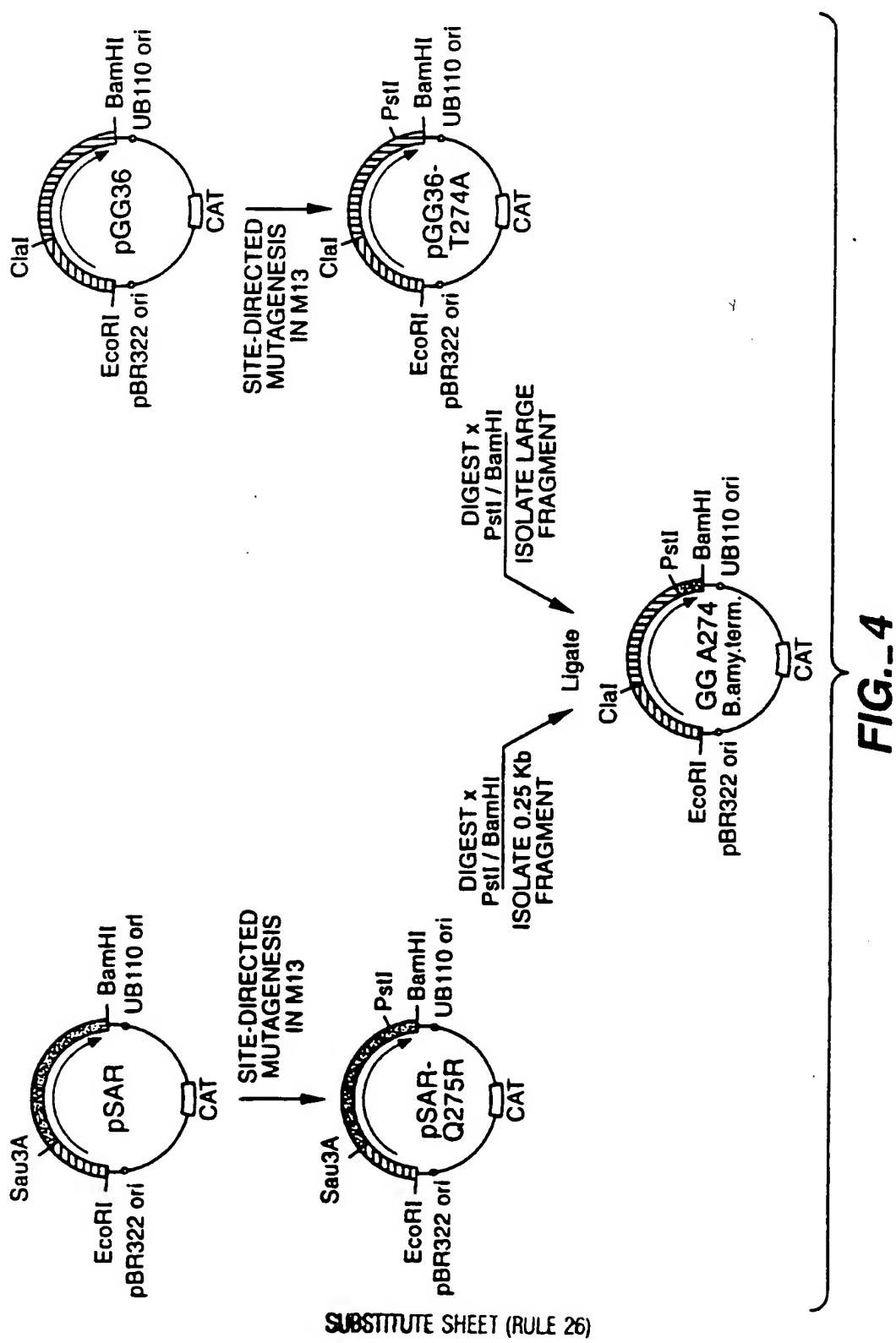
FIG. - 3B

FIG. 3

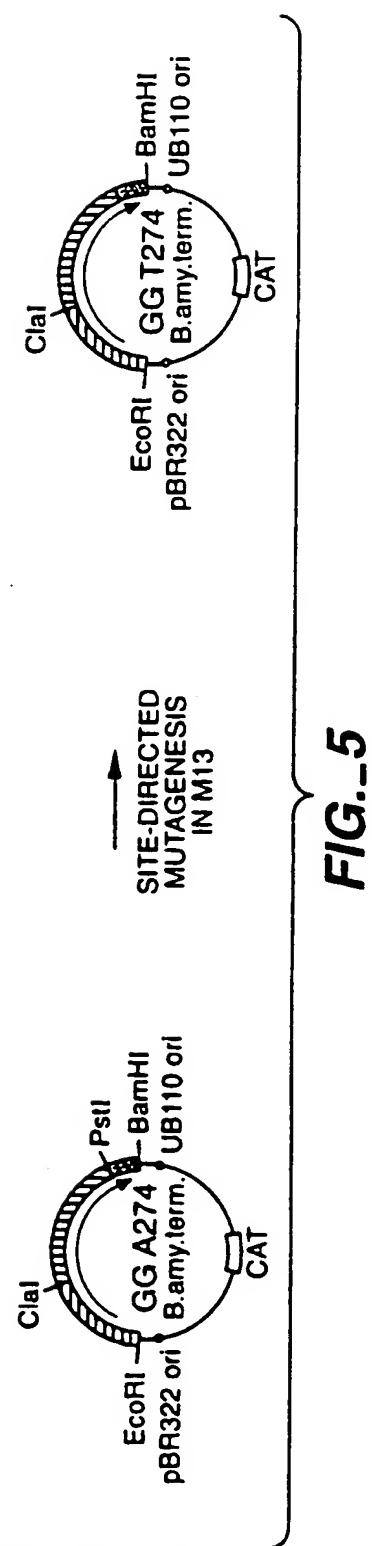
FIG.-3B

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**FIG. 5**

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FIG.—6A

FIG.—6B

850	870	890
GCAGCGGAGCTACTGACCAAAACAAACCAACCGGCCAGCTTTCACAGTATGGCCAGGG AlaValGlyAlaIleThrAspGlnAsnAlaSerGlnAsnArgAlaSerPheSerGlnIntyrglyAlaAlaGly		
910	930	950
CTTGCAATTGTCGCCACCACGCTGAAACGAGGACATACCCAGGTCAACGTATGCC LeuAlaIleValAlaProGlyValAlaSerValGlnSerThrTyrglySerThrTyrglyAla		
970	990	1010
AGCTTAACGGTACATCGATGGCTACTCCTCATGTTGCACGGTGCAGGCCCTGTGTTAA SerLeuSerGlyThrSerMetAlaThrProH1SerValAlaGlyAlaAlaLeuValLeu		
1030	1050	1070
CAAAACACCCATCTGGTCCAAATGTACAAATCCCAATCATCTAAAGAATACGGCAACG GlnLeuAlaProSerTrpSerAsnProSerGlnIleArgAsnH1SerValLeuValLeu		
1090	1110	1130
ACCTTAGGAGCCAACTTGTATGGCAAGGGGACTTGTCAATGGCAGAAGCCGAAACACGCC SerLeuGlySerThrAsnLeuTyrglySerGlyLeuValAlaAlaAlaLeuValLeu		

FIG.-6C

FIG.—6A

FIG.—6B

FIG. 6C

FIG. - 6

FIG.—6A

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10 ATGAGAACCGTTGGGAAATTGTCCAAAGCACCGCAACTCATTCTGCTTT
 MetLys**80** ProLeuGly**81** LeuAlaSerThrAlaLeuLeuSerValAlaPhe
 110 AGTCATC⁹⁰ ATGCCATGGCTGCTGAAGCAAAAGAAATTTAAATTGGCTTAAT
 SerSer**91** IleAlaSerAlaAlaGluAlaLys**92** GluLys**93** TyrLeuIleGlyPhe**94** Asn
 120 GACCCAGG**100** GCTCTCAGTCAGTTGAGAACAGTAGAGGCCAAATGACGAGGTCGCCATT
 Glu**101** Lys**102** Ala**103** Val**104** Ser**105** Glu**106** Val**107** Glu**108** Ile**109** Asn**110** Asp**111** Glu**112** Val**113** Ala**114** Val**115** Lys**116** Val**117** Ile**118** Leu**119** Lys**120** Val**121** Ile**122** Pro**123** Val**124** Glu**125** Ile**126** Glu**127** Val**128** Glu**129** Ile**130** Pro**131** Val**132** Glu**133** Ile**134** Glu**135** Val**136** Glu**137** Ile**138** Leu**139** Lys**140** Val**141** Ile**142** Pro**143** Val**144** Glu**145** Ile**146** Glu**147** Val**148** Glu**149** Ile**150** Pro**151** Val**152** Glu**153** Ile**154** Glu**155** Val**156** Glu**157** Ile**158** Leu**159** Lys**160** Val**161** Ile**162** Pro**163** Val**164** Glu**165** Ile**166** Glu**167** Val**168** Glu**169** Ile**170** Pro**171** Val**172** Glu**173** Ile**174** Glu**175** Val**176** Glu**177** Ile**178** Leu**179** Lys**180** Val**181** Ile**182** Pro**183** Val**184** Glu**185** Ile**186** Glu**187** Val**188** Glu**189** Ile**190** Pro**191** Val**192** Glu**193** Ile**194** Glu**195** Val**196** Glu**197** Ile**198** Leu**199** Lys**200** Val**201** Ile**202** Pro**203** Val**204** Glu**205** Ile**206** Glu**207** Val**208** Glu**209** Ile**210** Pro**211** Val**212** Glu**213** Ile**214** Glu**215** Val**216** Glu**217** Ile**218** Leu**219** Lys**220** Val**221** Ile**222** Pro**223** Val**224** Glu**225** Ile**226** Glu**227** Val**228** Glu**229** Ile**230** Pro**231** Val**232** Glu**233** Ile**234** Glu**235** Val**236** Glu**237** Ile**238** Leu**239** Lys**240** Val**241** Ile**242** Pro**243** Val**244** Glu**245** Ile**246** Glu**247** Val**248** Glu**249** Ile**250** Pro**251** Val**252** Glu**253** Ile**254** Glu**255** Val**256** Glu**257** Ile**258** Leu**259** Lys**260** Val**261** Ile**262** Pro**263** Val**264** Glu**265** Ile**266** Glu**267** Val**268** Glu**269** Ile**270** Pro**271** Val**272** Glu**273** Ile**274** Glu**275** Val**276** Glu**277** Ile**278** Leu**279** Lys**280** Val**281** Ile**282** Pro**283** Val**284** Glu**285** Ile**286** Glu**287** Val**288** Glu**289** Ile**290** Pro**291** Val**292** Glu**293** Ile**294** Glu**295** Val**296** Glu**297** Ile**298** Leu**299** Lys**300** Val**301** Ile**302** Pro**303** Val**304** Glu**305** Ile**306** Glu**307** Val**308** Glu**309** Ile**310** Pro**311** Val**312** Glu**313** Ile**314** Glu**315** Val**316** Glu**317** Ile**318** Leu**319** Lys**320** Val**321** Ile**322** Pro**323** Val**324** Glu**325** Ile**326** Glu**327** Val**328** Glu**329** Ile**330** Pro**331** Val**332** Glu**333** Ile**334** Glu**335** Val**336** Glu**337** Ile**338** Leu**339** Lys**340** Val**341** Ile**342** Pro**343** Val**344** Glu**345** Ile**346** Glu**347** Val**348** Glu**349** Ile**350** Pro**351** Val**352** Glu**353** Ile**354** Glu**355** Val**356** Glu**357** Ile**358** Leu**359** Lys**360** Val**361** Ile**362** Pro**363** Val**364** Glu**365** Ile**366** Glu**367** Val**368** Glu**369** Ile**370** Pro**371** Val**372** Glu**373** Ile**374** Glu**375** Val**376** Glu**377** Ile**378** Leu**379** Lys**380** Val**381** Ile**382** Pro**383** Val**384** Glu**385** Ile**386** Glu**387** Val**388** Glu**389** Ile**390** Pro**391** Val**392** Glu**393** Ile**394** Glu**395** Val**396** Glu**397** Ile**398** Leu**399** Lys**400** Val**401** Ile**402** Pro**403** Val**404** Glu**405** Ile**406** Glu**407** Val**408** Glu**409** Ile**410** Pro**411** Val**412** Glu**413** Ile**414** Glu**415** Val**416** Glu**417** Ile**418** Leu**419** Lys**420** Val**421** Ile**422** Pro**423** Val**424** Glu**425** Ile**426** Glu**427** Val**428** Glu**429** Ile**430** Pro**431** Val**432** Glu**433** Ile**434** Glu**435** Val**436** Glu**437** Ile**438** Leu**439** Lys**440** Val**441** Ile**442** Pro**443** Val**444** Glu**445** Ile**446** Glu**447** Val**448** Glu**449** Ile**450** Pro**451** Val**452** Glu**453** Ile**454** Glu**455** Val**456** Glu**457** Ile**458** Leu**459** Lys**460** Val**461** Ile**462** Pro**463** Val**464** Glu**465** Ile**466** Glu**467** Val**468** Glu**469** Ile**470** Pro**471** Val**472** Glu**473** Ile**474** Glu**475** Val**476** Glu**477** Ile**478** Leu**479** Lys**480** Val**481** Ile**482** Pro**483** Val**484** Glu**485** Ile**486** Glu**487** Val**488** Glu**489** Ile**490** Pro**491** Val**492** Glu**493** Ile**494** Glu**495** Val**496** Glu**497** Ile**498** Leu**499** Lys**500** Val**501** Ile**502** Pro**503** Val**504** Glu**505** Ile**506** Glu**507** Val**508** Glu**509** Ile**510** Pro**511** Val**512** Glu**513** Ile**514** Glu**515** Val**516** Glu**517** Ile**518** Leu**519** Lys**520** Val**521** Ile**522** Pro**523** Val**524** Glu**525** Ile**526** Glu**527** Val**528** Glu**529** Ile**530** Pro**531** Val**532** Glu**533** Ile**534** Glu**535** Val**536** Glu**537** Ile**538** Leu**539** Lys**540** Val**541** Ile**542** Pro**543** Val**544** Glu**545** Ile**546** Glu**547** Val**548** Glu**549** Ile**550** Pro**551** Val**552** Glu**553** Ile**554** Glu**555** Val**556** Glu**557** Ile**558** Leu**559** Lys**560** Val**561** Ile**562** Pro**563** Val**564** Glu**565** Ile**566** Glu**567** Val**568** Glu**569** Ile**570** Pro**571** Val**572** Glu**573** Ile**574** Glu**575** Val**576** Glu**577** Ile**578** Leu**579** Lys**580** Val**581** Ile**582** Pro**583** Val**584** Glu**585** Ile**586** Glu**587** Val**588** Glu**589** Ile**590** Pro**591** Val**592** Glu**593** Ile**594** Glu**595** Val**596** Glu**597** Ile**598** Leu**599** Lys**600** Val**601** Ile**602** Pro**603** Val**604** Glu**605** Ile**606** Glu**607** Val**608** Glu**609** Ile**610** Pro**611** Val**612** Glu**613** Ile**614** Glu**615** Val**616** Glu**617** Ile**618** Leu**619** Lys**620** Val**621** Ile**622** Pro**623** Val**624** Glu**625** Ile**626** Glu**627** Val**628** Glu**629** Ile**630** Pro**631** Val**632** Glu**633** Ile**634** Glu**635** Val**636** Glu**637** Ile**638** Leu**639** Lys**640** Val**641** Ile**642** Pro**643** Val**644** Glu**645** Ile**646** Glu**647** Val**648** Glu**649** Ile**650** Pro**651** Val**652** Glu**653** Ile**654** Glu**655** Val**656** Glu**657** Ile**658** Leu**659** Lys**660** Val**661** Ile**662** Pro**663** Val**664** Glu**665** Ile**666** Glu**667** Val**668** Glu**669** Ile**670** Pro**671** Val**672** Glu**673** Ile**674** Glu**675** Val**676** Glu**677** Ile**678** Leu**679** Lys**680** Val**681** Ile**682** Pro**683** Val**684** Glu**685** Ile**686** Glu**687** Val**688** Glu**689** Ile**690** Pro**691** Val**692** Glu**693** Ile**694** Glu**695** Val**696** Glu**697** Ile**698** Leu**699** Lys**700** Val**701** Ile**702** Pro**703** Val**704** Glu**705** Ile**706** Glu**707** Val**708** Glu**709** Ile**710** Pro**711** Val**712** Glu**713** Ile**714** Glu**715** Val**716** Glu**717** Ile**718** Leu**719** Lys**720** Val**721** Ile**722** Pro**723** Val**724** Glu**725** Ile**726** Glu**727** Val**728** Glu**729** Ile**730** Pro**731** Val**732** Glu**733** Ile**734** Glu**735** Val**736** Glu**737** Ile**738** Leu**739** Lys**740** Val**741** Ile**742** Pro**743** Val**744** Glu**745** Ile**746** Glu**747** Val**748** Glu**749** Ile**750** Pro**751** Val**752** Glu**753** Ile**754** Glu**755** Val**756** Glu**757** Ile**758** Leu**759** Lys**760** Val**761** Ile**762** Pro**763** Val**764** Glu**765** Ile**766** Glu**767** Val**768** Glu**769** Ile**770** Pro**771** Val**772** Glu**773** Ile**774** Glu**775** Val**776** Glu**777** Ile**778** Leu**779** Lys**780** Val**781** Ile**782** Pro**783** Val**784** Glu**785** Ile**786** Glu**787** Val**788** Glu**789** Ile**790** Pro**791** Val**792** Glu**793** Ile**794** Glu**795** Val**796** Glu**797** Ile**798** Leu**799** Lys**800** Val**801** Ile**802** Pro**803** Val**804** Glu**805** Ile**806** Glu**807** Val**808** Glu**809** Ile**810** Pro**811** Val**812** Glu**813** Ile**814** Glu**815** Val**816** Glu**817** Ile**818** Leu**819** Lys**820** Val**821** Ile**822** Pro**823** Val**824** Glu**825** Ile**826** Glu**827** Val**828** Glu**829** Ile**830** Pro**831** Val**832** Glu**833** Ile**834** Glu**835** Val**836** Glu**837** Ile**838** Leu**839** Lys**840** Val**841** Ile**842** Pro**843** Val**844** Glu**845** Ile**846** Glu**847** Val**848** Glu**849** Ile**850** Pro**851** Val**852** Glu**853** Ile**854** Glu**855** Val**856** Glu**857** Ile**858** Leu**859** Lys**860** Val**861** Ile**862** Pro**863** Val**864** Glu**865** Ile**866** Glu**867** Val**868** Glu**869** Ile**870** Pro**871** Val**872** Glu**873** Ile**874** Glu**875** Val**876** Glu**877** Ile**878** Leu**879** Lys**880** Val**881** Ile**882** Pro**883** Val**884** Glu**885** Ile**886** Glu**887** Val**888** Glu**889** Ile**890** Pro**891** Val**892** Glu**893** Ile**894** Glu**895** Val**896** Glu**897** Ile**898** Leu**899** Lys**900** Val**901** Ile**902** Pro**903** Val**904** Glu**905** Ile**906** Glu**907** Val**908** Glu**909** Ile**910** Pro**911** Val**912** Glu**913** Ile**914** Glu**915** Val**916** Glu**917** Ile**918** Leu**919** Lys**920** Val**921** Ile**922** Pro**923** Val**924** Glu**925** Ile**926** Glu**927** Val**928** Glu**929** Ile**930** Pro**931** Val**932** Glu**933** Ile**934** Glu**935** Val**936** Glu**937** Ile**938** Leu**939** Lys**940** Val**941** Ile**942** Pro**943** Val**944** Glu**945** Ile**946** Glu**947** Val**948** Glu**949** Ile**950** Pro**951** Val**952** Glu**953** Ile**954** Glu**955** Val**956** Glu**957** Ile**958** Leu**959** Lys**960** Val**961** Ile**962** Pro**963** Val**964** Glu**965** Ile**966** Glu**967** Val**968** Glu**969** Ile**970** Pro**971** Val**972** Glu**973** Ile**974** Glu**975** Val**976** Glu**977** Ile**978** Leu**979** Lys**980** Val**981** Ile**982** Pro**983** Val**984** Glu**985** Ile**986** Glu**987** Val**988** Glu**989** Ile**990** Pro**991** Val**992** Glu**993** Ile**994** Glu**995** Val**996** Glu**997** Ile**998** Leu**999** Lys**1000** Val**1001** Ile**1002** Pro**1003** Val**1004** Glu**1005** Ile**1006** Glu**1007** Val**1008** Glu**1009** Ile**1010** Pro**1011** Val**1012** Glu**1013** Ile**1014** Glu**1015** Val**1016** Glu**1017** Ile**1018** Leu**1019** Lys**1020** Val**1021** Ile**1022** Pro**1023** Val**1024** Glu**1025** Ile**1026** Glu**1027** Val**1028** Glu**1029** Ile**1030** Pro**1031** Val**1032** Glu**1033** Ile**1034** Glu**1035** Val**1036** Glu**1037** Ile**1038** Leu**1039** Lys**1040** Val**1041** Ile**1042** Pro**1043** Val**1044** Glu**1045** Ile**1046** Glu**1047** Val**1048** Glu**1049** Ile**1050** Pro**1051** Val**1052** Glu**1053** Ile**1054** Glu**1055** Val**1056** Glu**1057** Ile**1058** Leu**1059** Lys**1060** Val**1061** Ile**1062** Pro**1063** Val**1064** Glu**1065** Ile**1066** Glu**1067** Val**1068** Glu**1069** Ile**1070** Pro**1071** Val**1072** Glu**1073** Ile**1074** Glu**1075** Val**1076** Glu**1077** Ile**1078** Leu**1079** Lys**1080** Val**1081** Ile**1082** Pro**1083** Val**1084** Glu**1085** Ile**1086** Glu**1087** Val**1088** Glu**1089** Ile**1090** Pro**1091** Val**1092** Glu**1093** Ile**1094** Glu**1095** Val**1096** Glu**1097** Ile**1098** Leu**1099** Lys**1100** Val**1101** Ile**1102** Pro**1103** Val**1104** Glu**1105** Ile**1106** Glu**1107** Val**1108** Glu**1109** Ile**1110** Pro**1111** Val**1112** Glu**1113** Ile**1114** Glu**1115** Val**1116** Glu**1117** Ile**1118** Leu**1119** Lys**1120** Val**1121** Ile**1122** Pro**1123** Val**1124** Glu**1125** Ile**1126** Glu**1127** Val**1128** Glu**1129** Ile**1130** Pro**1131** Val**1132** Glu**1133** Ile**1134** Glu**1135** Val**1136** Glu**1137** Ile**1138** Leu**1139** Lys**1140** Val**1141** Ile**1142** Pro**1143** Val**1144** Glu**1145** Ile**1146** Glu**1147** Val**1148** Glu**1149** Ile**1150** Pro**1151** Val**1152** Glu**1153** Ile**1154** Glu**1155** Val**1156** Glu**1157** Ile**1158** Leu**1159** Lys**1160** Val**1161** Ile**1162** Pro**1163** Val**1164** Glu**1165** Ile**1166** Glu**1167** Val**1168** Glu**1169** Ile**1170** Pro**1171** Val**1172** Glu**1173** Ile**1174** Glu**1175** Val**1176** Glu**1177** Ile**1178** Leu**1179** Lys**1180** Val**1181** Ile**1182** Pro**1183** Val**1184** Glu**1185** Ile**1186** Glu**1187** Val**1188** Glu**1189** Ile**1190** Pro**1191** Val**1192** Glu**1193** Ile**1194** Glu**1195** Val**1196** Glu**1197** Ile**1198** Leu**1199** Lys**1200** Val**1201** Ile**1202** Pro**1203** Val**1204** Glu**1205** Ile**1206** Glu**1207** Val**1208** Glu**1209** Ile**1210** Pro**1211** Val**1212** Glu**1213** Ile**1214** Glu**1215** Val**1216** Glu**1217** Ile**1218** Leu**1219** Lys**1220** Val**1221** Ile**1222** Pro**1223** Val**1224** Glu**1225** Ile**1226** Glu**1227** Val**1228** Glu**1229** Ile**1230** Pro**1231** Val**1232** Glu**1233** Ile**1234** Glu**1235** Val**1236** Glu**1237** Ile**1238** Leu**1239** Lys**1240** Val**1241** Ile**1242** Pro**1243** Val**1244** Glu**1245** Ile**1246** Glu**1247** Val**1248** Glu**1249** Ile**1250** Pro**1251** Val**1252** Glu**1253** Ile**1254** Glu**1255** Val**1256** Glu**1257** Ile**1258** Leu**1259** Lys**1260** Val**1261** Ile**1262** Pro**1263** Val**1264** Glu**1265** Ile**1266** Glu**1267** Val**1268** Glu**1269** Ile**1270** Pro**1271** Val**1272** Glu**1273** Ile**1274** Glu**1275** Val**1276** Glu**1277** Ile**1278** Leu**1279** Lys**1280** Val**1281** Ile**1282** Pro**1283** Val**1284** Glu**1285** Ile**1286** Glu**1287** Val**1288** Glu**1289** Ile**1290** Pro**1291** Val**1292** Glu**1293** Ile**1294** Glu**1295** Val**1296** Glu**1297** Ile**1298** Leu**1299** Lys**1300** Val**1301** Ile**1302** Pro**1303** Val**1304** Glu**1305** Ile**1306** Glu**1307** Val**1308** Glu**1309** Ile**1310** Pro**1311** Val**1312** Glu**1313** Ile**1314** Glu**1315** Val**1316** Glu**1317** Ile**1318** Leu**1319** Lys**1320** Val**1321** Ile**1322** Pro**1323** Val**1324**

FIG. - 7A

430	450	470
CTCCTCGATACTAGCTATTCCACTCATCCAGACTAAATTCGCTGCCCTACGCCIT		
ValLeuAspThrGlyIleSerThrHisProAspPleAsnIleArgGlyAlaSerPhe		
490	510	530
GTACCAAGGGAAACCATCCACTCAAGATGGGAATGGGCATGCCACCCATGTCGCCGAGC		
ValProGlyGluProSerThrGlnAspGlyAsnGlyIleGlyThrHisValAlaGlyThr		
550	570	590
ATGGCTGCTTTAGACAACTCGATTGCCGTTCTGGCCTAGGCCGAGCCCCGAACTATAC		
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr		
610	630	650
GCTGTTAAGTATTAGGGCGAGCCGCTTCAGGCCCATCAGCTCGATTGCCCAAGGATTG		
AlaValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu		
670	690	710
GAATGGCAGGGAAACAATGCCATGCCACGTTGCTATTGAGTTAGCAAGGCCCTGGCCA		
GlutTrpAlaGlyAsnGlyAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro		
730	750	770
AGTGCCACACTTGAGCAAGCTGTTAATAGGCCGACTTCTAGAGGCCGTTCTTGTTGAGCG		
SerAlaThrLeuGluGlnAlaValAlaAsnSerAlaThrSerArgGlyValLeuValAlaIle		
790	810	830
GCATCTGGAAATTCAAGCTGCAGGCCCAATCAGCTATCCGGCCGTTATGCCAACGGCAATG		
AlaSerGlyAsnSerGlyAlaGlySerIleSerIleArgTyrAlaAsnAlaMet		

FIG._7B

850	870	890
GCAGTCGGAGCTACTGACCAAAACAACAA	ACCGGCCAGCTTTCACAGTATGGCCAGGG	
AlaValGlyAlaIleThrAspGlnAsnAlaSerPheSerGlnTyrGlyAlaGly		
910	930	950
CTTGACATTGTGCCACCACCTGTAACCTGAGCACATACCCAGCTAACGTTAACGTTGCC		
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla		
970	990	1010
AGCTTAAACGGCTACATCGATGGCTACTCCATGTCAGGCTGCAGGCCCTGTAAA		
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaLeuValAla		
1030	1050	1070
CAAAAGAACCCATCTTGGCCAATGTACAAATCCC	AAATCATCTAAAGAAATACGGCAAACG	
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuIleAsnThrAlaThr		
1090	1110	1130
ACCTTAGGAAAGCACGAACTTGTATGGAAACCCGACTTGTCAATGCCAGAAGGGCCAAACACGC		
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaCuaAlaAlaThrArg		

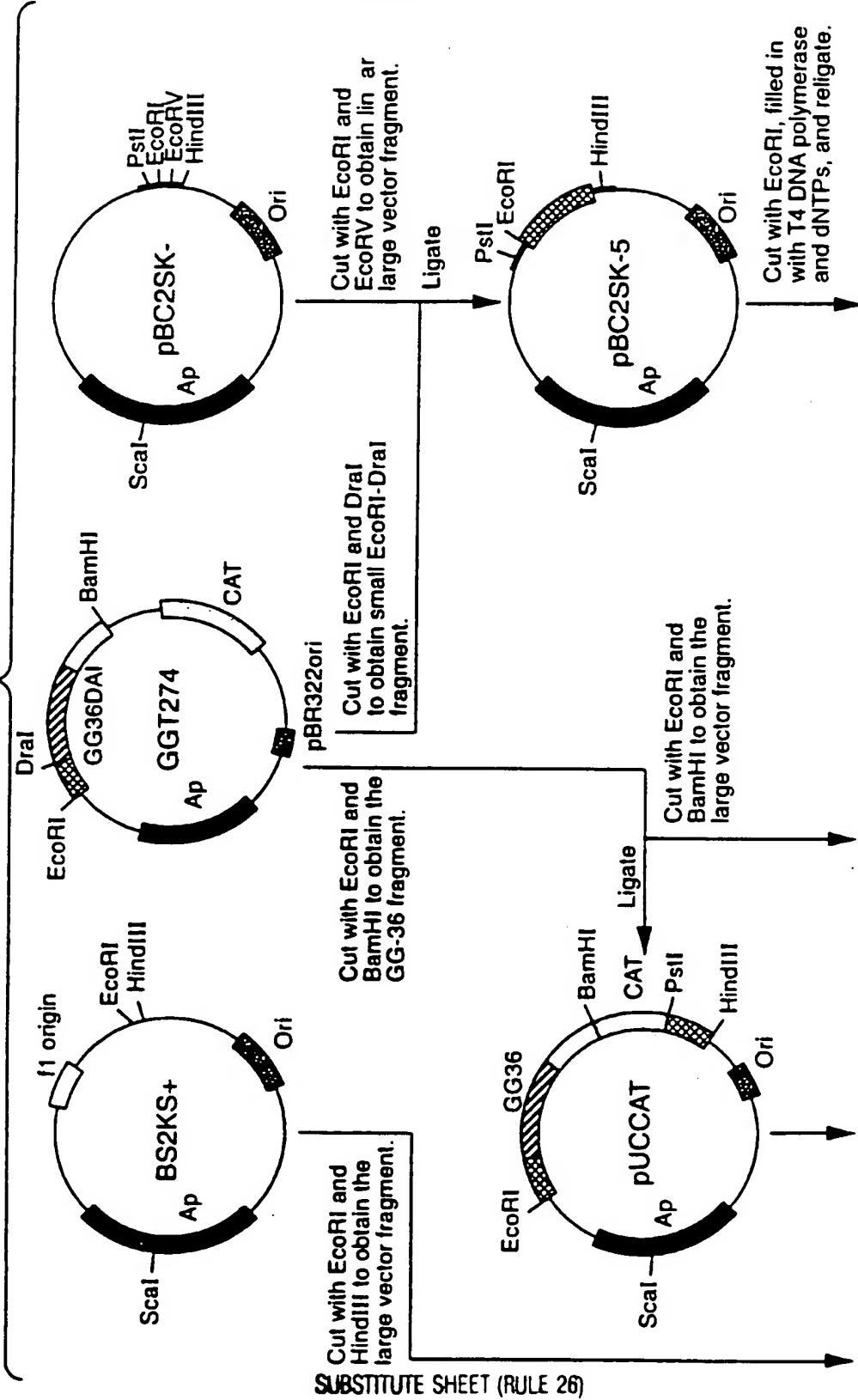
FIG.-7C

FIG.-7A

FIG.-7B

FIG.-7

FIG. - 8A



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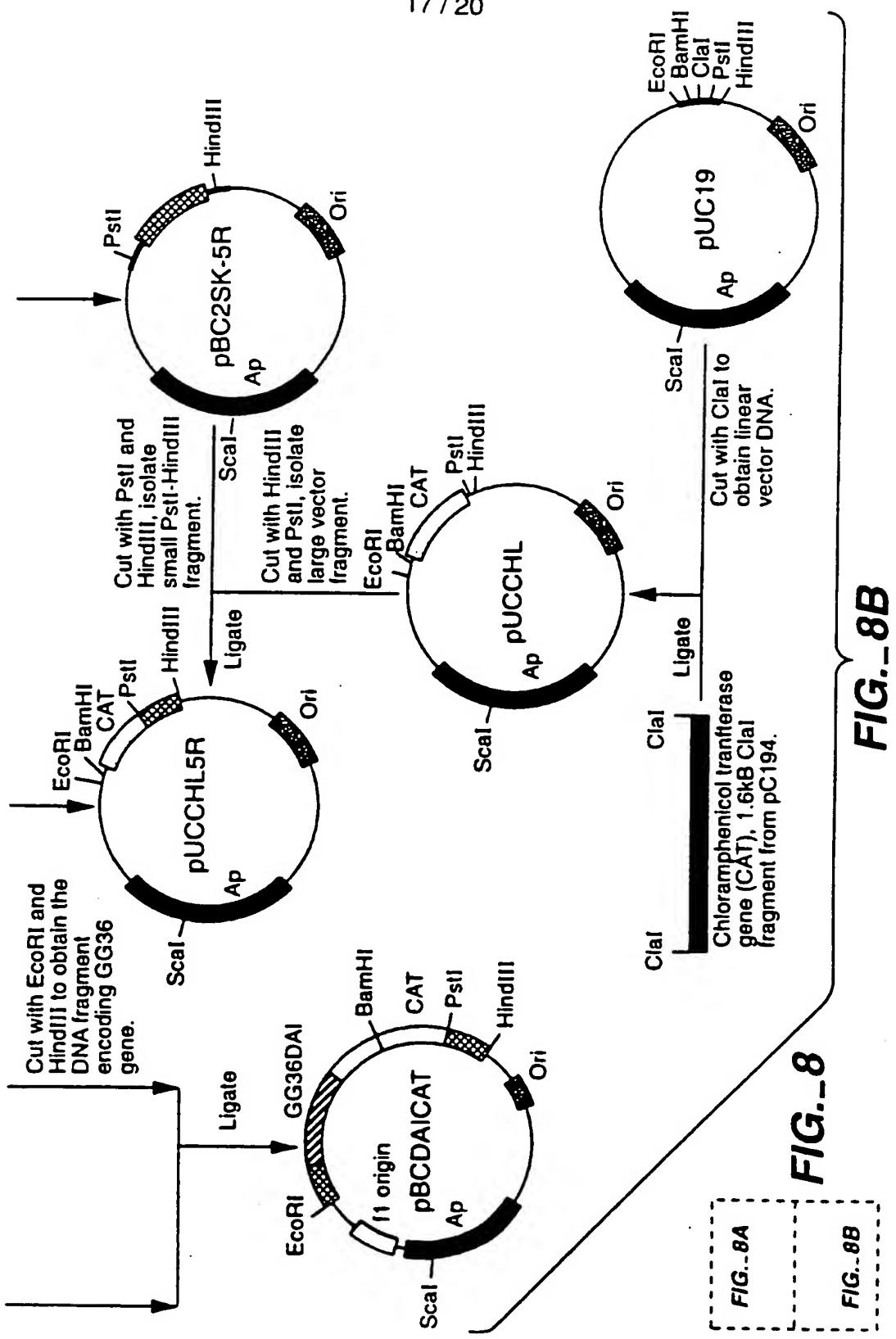
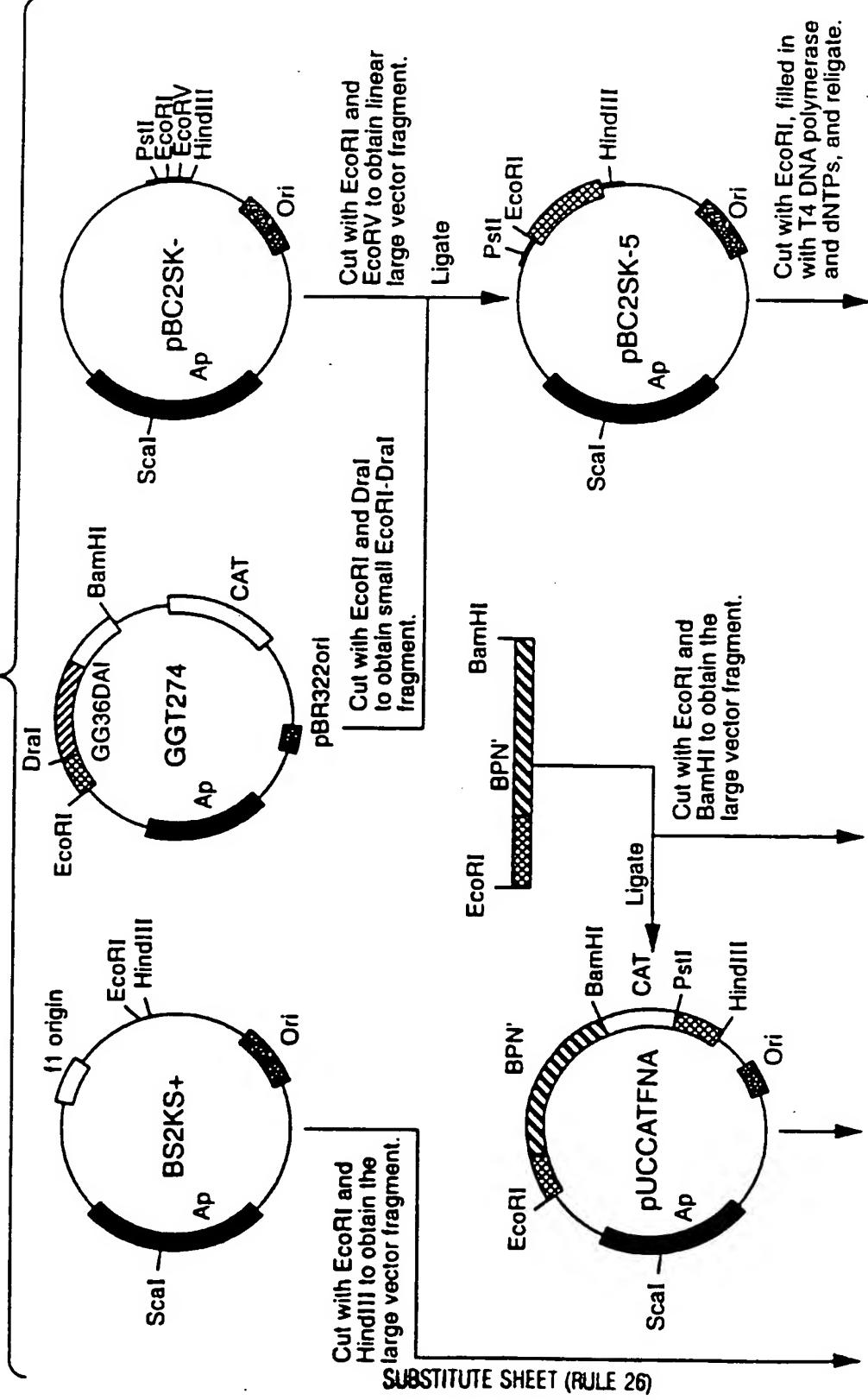


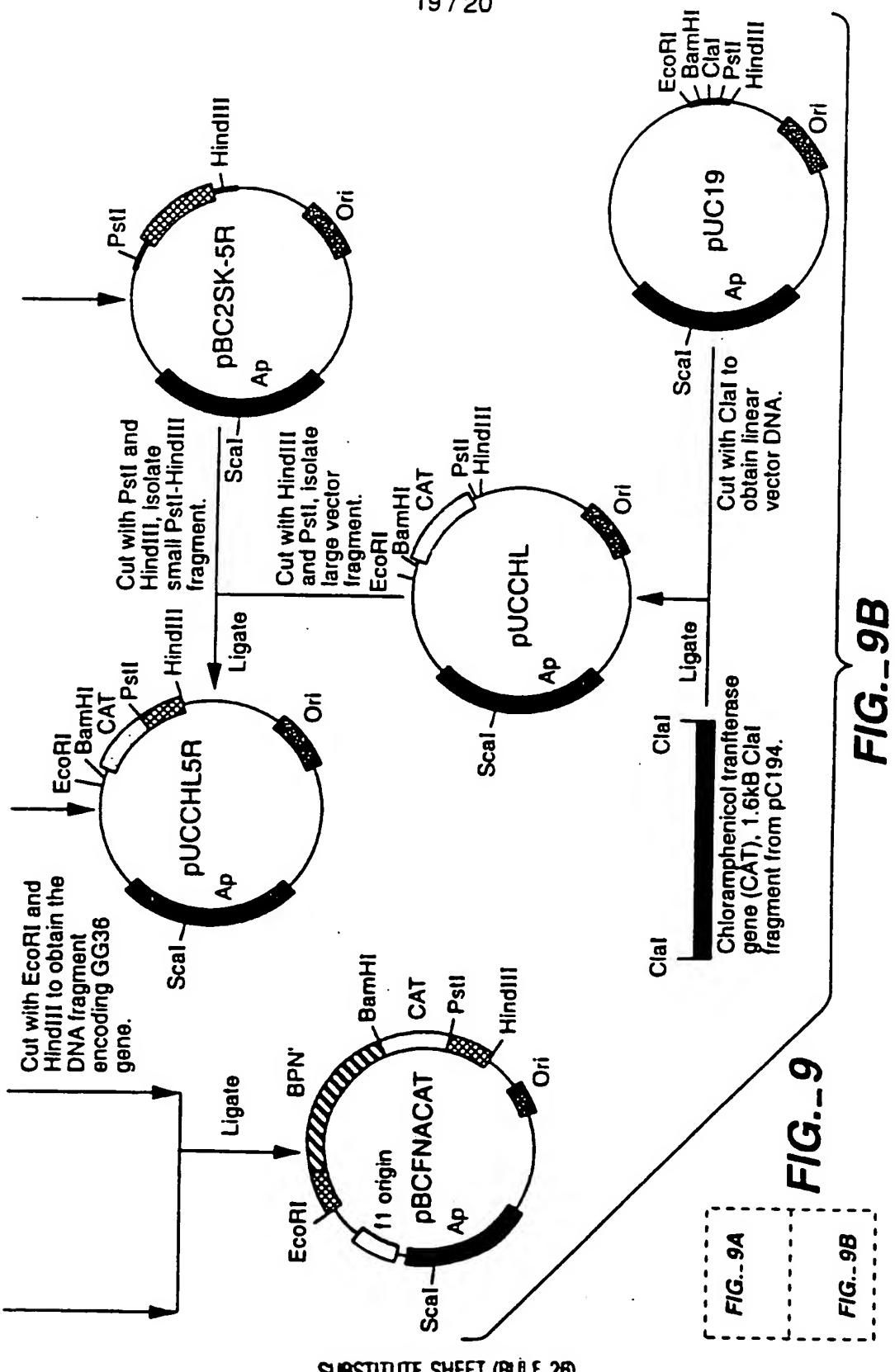
FIG. 8

FIG. 8A

FIG. 8B

FIG. 9A





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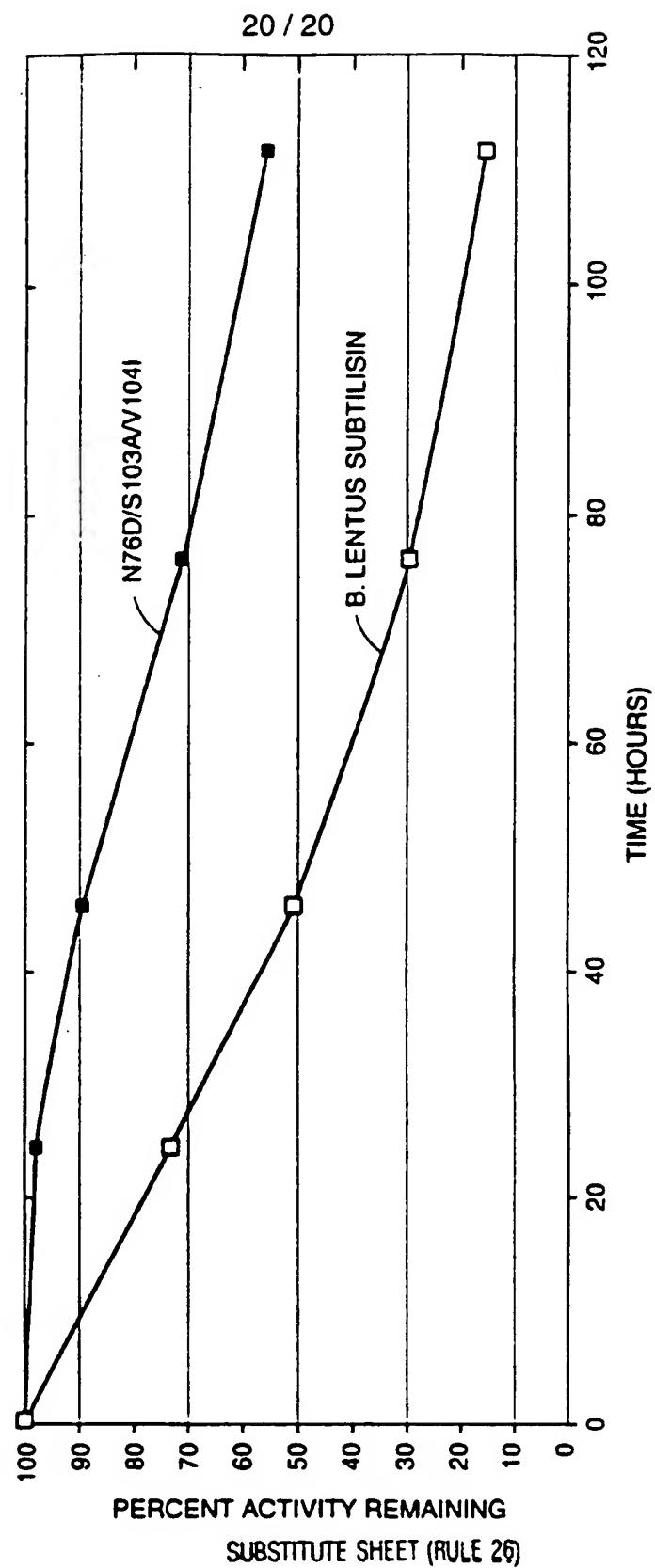


FIG._10

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C11D3/386 A61K7/48 C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C11D C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 398 539 (AMGEN INC.) 22 November 1990 see page 7, line 14 - line 43; claims 1-23; examples ---	1-21
X	EP,A,0 405 901 (UNILEVER) 2 January 1991 see page 30, line 25 - page 35, line 47; claims; examples ---	1-21
X	WO,A,88 08033 (AMGEN INC.) 20 October 1988 see page 14, line 1 - page 15, line 30; claims ---	1-21
X	WO,A,89 09830 (GENEX CORP.) 19 October 1989 see claims 11-16 ---	1-21 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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*'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

*'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

16 February 1995

Date of mailing of the international search report

- 8.03.95

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Grittern, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO,A,91 06637 (GENENCOR INT.) 16 May 1991 cited in the application see the whole document ----	1
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Information on patent family members

Int'l Application No

PCT/US 94/11776

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